Proton Magnetic Resonance Studies of Horse Cytochrome *c*†

C. C. McDonald and W. D. Phillips*

ABSTRACT: Extensive regions of the proton magnetic resonance (pmr) spectra (220 MHz) of horse ferro- and ferricytochrome *c* were studied as a function of pH, temperature, and solvent environment. Native ferrocytochrome *c* exhibits 20 resolved single CH and CH_3 resonances and 3 resolved NH resonances at the high- and low-field extremes of the spectrum as well as many partially resolved resonances in the central overlapped region. Resonances of His-18, Met-80 (heme iron ligands), and porphyrin meso proteins are firmly assigned; other resolved resonances are tentatively assigned to His-26, His-33, Leu-32, Trp-59, Thr-63, Ile-75, and the thioether bridge methyl of porphyrin ring 1. Position of many resonances of the native reduced protein are shifted from primary resonance positions because of ring-current fields and "solvent" shifts. Major elements of the conformation of ferrocytochrome *c* are conserved from pH 4.5 to 10 and from 5 to 56°. There is evidence for a minor temperature-dependent conformational change through this temperature interval. Denaturation to an extended form occurs at higher temperatures. Interior peptide NH protons of native ferrocytochrome *c* in D_2O (neutral solution, 23°) exchange very slowly, indicating little unfolding of the protein; one region is particularly stable. On oxidation of cytochrome *c* to the native paramagnetic ferri form, the pmr spectrum is greatly altered mainly because of introduction of large hyperfine contact-shift perturbations to resonances of protons associated with the heme group, His-18, and Met-80 and substantial pseudo-contact-shift perturbations of resonances of protons near the heme iron. Fifteen contact-shifted resonances are resolved outside the central region; assignments of 14 are discussed. Effects of temperature and pH on 15 resolved and 63 partially resolved resonances were measured. Temperature shifts appear to be associated mainly with contact-shifted resonances but several resonances exhibited marked departures from Curie law behavior. Major conformational features of native ferricytochrome *c* are conserved up to 55° and from pH 5 to 9. Labile protons of ferricytochrome *c* exchange readily in neutral D_2O at room temperature except for a small number in a region of tertiary structure (the most stable region of ferrocytochrome *c*) that unfolds when heated in mildly alkaline solution. No evidence of exchange of porphyrin meso protons was observed when cytochrome *c* in D_2O was cycled between the oxidized and reduced states.

Cytochrome *c* is a globular protein found in mitochondria of aerobic tissues. It functions in respiration by transferring electrons from cytochrome *b* to cytochrome oxidase. The constituents of cytochrome *c* are a heme prosthetic group (Figure 1) and a polypeptide of about 100 amino acid residues that varies somewhat in composition and length for different phylogenetic species. The heme is attached to the polypeptide *via* thioether linkages from porphyrin substituents to two cysteine residues and by coordination of histidine and methionine residues to the heme iron atom at the fifth and sixth (axial) ligand positions, respectively. The protein exhibits stable oxidized and reduced forms. In the oxidized or ferri form the heme iron is in the paramagnetic low-spin Fe^{3+} ($S = {}^1/_2$) state; in the reduced or ferro form the iron is diamagnetic low-spin Fe^{2+} ($S = 0$).

Cytochromes *c* from eukaryotic organisms have been studied extensively by chemical and physical methods and much has been learned about the structures and interactions of these important electron-transport proteins (Margoliash and Schejter, 1966; Dickerson and Geis, 1969; Kamen *et al.*, 1971; Chance *et al.*, 1966). Horse cytochrome *c* has been examined in the most detail. Recently atomic coordinates of crystalline horse ferricytochrome *c* determined by X-ray diffraction (Dickerson *et al.*, 1971) have revealed the nature of the folding of the polypeptide chain and its relationship to the heme group. Relatively little is known about the mechanisms of the biological functions of cytochrome *c*. Much current investigation is devoted to elucidation of the changes in conformation and electronic state that occur when the protein interacts with other molecules. Since cytochromes *c* from different eukaryotic species differ considerably in amino acid composition, other research is aimed at determining the relationship between composition and the conformation and chemistry of the protein. Finally, *c*-type cytochromes have been found in prokaryotic organisms that have some characteristics in common with eukaryotic cytochromes *c* but also exhibit important differences. A complete understanding of the dependence of function of cytochromes *c* on composition and structure will need to include consideration of proteins from prokaryotic organisms. The characteristics of proton magnetic resonance (pmr) spectra of native proteins in aqueous solution are usually dependent on protein tertiary structure and can provide information on subtle conformational changes that may accompany protein interactions or changes in protein composition (McDonald and Phillips, 1970, and references therein). Consequently, pmr spectroscopy has potential utility to contribute further elucidation of the relationship of cytochrome *c* structure and chemistry to biological function.

Several pmr studies of cytochrome *c* have been reported. The first (Kowalsky, 1962) was an examination of horse cytochrome *c* in neutral D_2O at a spectrometer frequency of 56.4 MHz. An envelope of overlapped resonances was observed extending from −3.2 to 10 ppm.[1] The strongest regions

† Contribution No. 2011 from the Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898. *Received March 9, 1973.*

[1] Literature resonance positions reported herein refer to the methyl resonance of the sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid used as an internal reference; positive shifts are to low field from the reference resonance.

of resonance absorption could be accounted for from the amino acid composition of the protein and the expected proton resonance positions of the amino acid residues. Weak resonance absorption from 0 to −3.2 ppm, to high field from expected resonance positions, was tentatively attributed to protons of residues near the faces of the porphyrin moiety and therefore subject to ring-current magnetic fields that shift resonances to high field. Some differences were detected between the resonance patterns of ferricytochrome *c* and ferrocytochrome *c* that could arise from different conformations and/or different magnetic properties of the two states. A later study (Kowalsky, 1965) on horse ferricytochrome *c* revealed three resonances at 31, 34, and −24 ppm far outside the normal spectral region (0–10 ppm). These large shifts were attributed to hyperfine contact interactions between certain protein protons and delocalized electron spin density from the heme iron atom. The three contact-shifted resonances were constant from pD 5 to 9 but at pD 10.4 they were replaced by contact-shifted resonances at 13.0, 20.9, and 23.3 ppm, implying a new state or conformation of the protein. No contact-shifted resonances were observed at pD values of 1.4 or 3.1. Denatured ferricytochrome *c* in 8 M urea exhibited no contact-shifted resonances and resonance absorption was confined to the normal region. Kowalsky also showed that the exchange rate between ferricytochrome *c* and ferrocytochrome *c* was $<10^5$ M sec^{-1} since positions of the contact-shifted resonances were not affected by ferrocytochrome *c*.

Next the normal region of horse ferricytochrome *c* was examined with a 100-MHz spectrometer (Mandel, 1965) but resolution of the spectrum was improved little over the earlier work. In 1967, we reported that the pmr spectra of horse cytochrome *c* exhibited much greater detail if acquired at 220 MHz (McDonald and Phillips, 1967). Completely resolved resonances were observed in the aromatic and near high-field regions as well as improved definition in the strongly overlapped region from 0 to 3 ppm. The spectra of ferricytochrome *c* and ferrocytochrome *c* were clearly quite different from that of the thermally denatured protein (neutral 5 M urea solution at 65°) and from each other. Slow exchange of the oxidized and reduced states was confirmed from observations that a solution containing both forms exhibited a spectrum containing resonances characteristic of each state. Almost all subsequent work on cytochrome *c* has been performed at 220 MHz.

Four completely resolved resonances in the high-field region of horse ferrocytochrome *c* between −1.5 and −4.0 ppm were assigned to a methionine residue positioned very close to the face of the porphyrin ring so that the protons are in a very strong ring-current magnetic field (McDonald and Phillips, 1968, 1969). Clearly, the resonances were associated with methionine at the sixth ligand position of the heme iron. Earlier chemical and X-ray studies had implicated Met-80 as this ligand. These spectral features were identical in pmr spectra of ferrocytochromes *c* from eight other eukaryotic species. More recently (McDonald *et al.*, 1970; Krejcarek *et al.*, 1971) similar resonances of a methionine heme ligand have been observed for reduced cytochromes *c* from several bacteria (*Pseudomonas aeruginosa*, *Rhodospirillum rubrum*, *Rhodopseudomonas capsulatus*, and *Rhodospirillum molischianum*) and a diatom (*Navicula pelliculosa*).

Wüthrich has studied the contact-shifted resonances of ferricytochrome *c* (Wüthrich, 1969, 1970, 1971). These resonances appeared identical for several eukaryotic species (horse, turkey, rabbit, and guanaco) but were somewhat different for ferricytochrome *c* from yeast (*Candida crusei*).

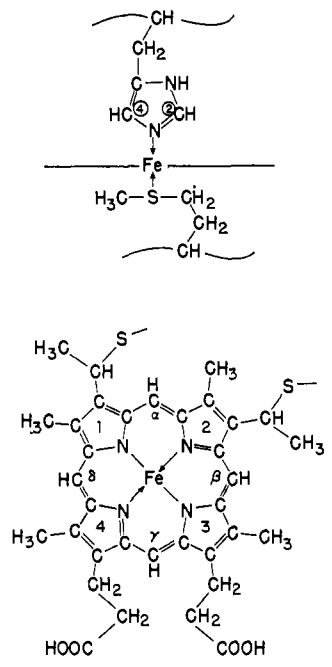

FIGURE 1: Heme prosthetic group of cytochrome *c*.

Eight single-proton resonances and six methyl resonances were resolved beyond the normal spectral region and were attributed to protons of the porphyrin ring, substituents on the porphyrin ring, and the histidine and methionine ligands of the heme iron. The large shifts of these resonances from their normal positions were attributed to hyperfine contact interactions; pseudocontact shifts were concluded to be small or absent. The contact-shifted resonances at −23.2, 31.4, and 34.0 (pD 7, 35°) originally detected by Kowalsky were assigned to the methyl resonance of Met-80 and to two of the methyl substituents of the porphyrin ring, respectively. Gupta and Redfield devised an elegant double-resonance modification of a pmr spectrometer operating in the Fourier transform mode that permits one to correlate the resonance positions of particular protons in the spectra of ferri- and ferrocytochrome *c* (Gupta and Redfield, 1970a). They have confirmed the assignments of the Met-80 methyl resonance of ferri- and ferrocytochrome *c* and the lowest field contact-shifted methyl resonances of ferricytochrome *c* to two porphyrin methyl groups. The resonance positions of the other two porphyrin methyls were identified in the spectrum of ferricytochrome *c* and the resonance positions of all four porphyrin methyls were detected for the reduced proton. Two contact-shifted resonances at −2.1 and −2.6 ppm (35°) of ferricytochrome *c* were assigned tentatively to methyl groups associated with the heme thioether linkage or to amino acid residues close to the edge of the porphyrin ring. Additional assignments from these authors are discussed below.

In the low-field spectrum of ferrocytochrome *c* resonances at 9.02, 9.29, and 9.58 ppm have been assigned to heme meso CH protons and a single resonance that shifts with pD has been assigned to the C-2 proton of a histidine residue (McDonald and Phillips, 1970). The effect of pH on the contact-shifted resonances of ferricytochrome *c* was studied at 100 MHz (Gupta and Koenig, 1971). The spectrum of the protein in neutral solution persists from about pH 4 to 9. Two new forms of the protein form in more alkaline solutions ($pK \simeq$ 9 and 11) with new contact-shifted resonance positions. These appear to have low-spin ferric heme irons but do not have Met-80 as a ligand. Below pH 3 no contact shifts are

TABLE I: Inventory and Primary Resonance Positions of Horse Cytochrome *c* Protons.

Proton Type	Residue Type and Multiplicity	No. of Protons	Primary Resonance Position[a]
Aliphatic side-chain CH			
CH_3	Leu (6)	36	0.89
	Ile (6)	36	0.83
	Val (3)	18	0.93
	Ala (6)	18	1.41
	Thr (10)	30	1.23
	Met (2)	6	2.06
		144 (48 methyl groups)	
Other aliphatic	Leu (6) β-CH_2 + γ-CH	18	1.64
	Ile (6) CH_2	12	1.13–1.41
	CH	6	~1.93
	Val (3) CH	3	2.25
	Thr (10) CH	10	~4.22
	Lys (19) γ-CH_2	38	1.43
	δ-CH_2 + β-CH_2	76	1.68
	ϵ-CH_2	38	3.02
	Arg (2) γ-CH_2	4	1.66
	β-CH_2	4	1.84
	δ-CH_2	4	3.20
	Pro (4) γ-CH_2	8	2.02
	β-CH_2	8	2.12
	δ-CH_2	8	~3.30
	Glu (9) β-CH_2	18	1.98
	γ-CH_2	18	2.27
	Glu (3) β-CH_2	6	2.07
	γ-CH_2	6	2.32
	Asp (3) CH_2	6	2.68
	Asn (5) CH_2	10	2.79–2.90
	Met (2) β-CH_2	4	2.06
	γ-CH_2	4	~2.57
	Cys (2) CH_2	4	3.02
	His (3) CH_2	6	~3.18
	Phe (4) CH_2	8	2.96–3.18
	Tyr (4) CH_2	8	2.98
	Trp (1) CH_2	2	3.38
		337	
Aromatic side-chain CH	His (3) C-4	3	7.07
	C-2	3	7.91
	Phe (4)	20	7.26
	Tyr (4) ortho to OH	8	6.82
	meta to OH	8	7.09
	Trp (1) C-2	1	7.20
	C-5, C-6	2	7.04–7.12
	C-4, C-7	2	7.44–7.53
		47	
α-CH	All residues	116	~3.6–4.6
Heme CH	Porphyrin ring CH_3	12	~3.6
	CH(S)C*H*$_3$	6	~2.1
	C*H*$_2$$CH_2COO$	4	~4.6
	CH_2C*H*$_2$COO	4	~3.5
	Meso CH	4	~10.5
	C*H*(S)CH_3	2	~4.0
		32	
Total CH		676	
NH protons			
Amide NH	Peptide bonds	99	~7.7–9.1
	Gln (3)	6	
	Asn (5)	10	

TABLE I (*Continued*)

Proton Type	Residue Type and Multiplicity	No. of Protons	Primary Resonance Position[a]
Amine	Terminal	3	
	Lys (19)	57	7.5
Other	Arg (2)	10	~6.6 (3), 7.2 (1), and 7.5 (1)
	His (3)	3	
	Trp (1)	1	~10.2
Total NH		189	
OH protons	Thr (10)	10	
	Tyr (4)	4	

[a] Parts per million from the sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid.

observed, presumably because the heme iron has converted to the high-spin ferric state.

A number of studies of pmr spectra (mainly of the contact-shifted resonances) of chemically modified cytochromes *c* have been made. These include cyanoferricytochrome *c* (McDonald and Phillips, 1967; Wüthrich, 1969), azidoferricytochrome *c* (Wüthrich, 1971; Gupta and Redfield, 1970b), ferricytochrome *c* carboxymethylated at Met-65 or at Met-65 and Met-80 (McDonald and Phillips, 1970; Wüthrich, 1971), and ferricytochrome *c* formylated at Trp-59 (Wüthrich, 1971).

Several steps are involved in obtaining biological information from protein pmr spectra. First, one must acquire spectra of the native protein in aqueous solution that exhibit an adequate number of resolved individual resonances. Next, one attempts to assign these resonances to particular protons in the protein molecule. Finally, biological information is obtained through rationalization of resonance characteristics in terms of protein structure and interactions. None of these steps is straightforward for cytochrome *c* but advances have been made in all aspects in the several pmr studies that have been reported. Both the quality of the spectra of cytochrome *c* and the biological information derived from them have increased markedly as the pmr technique has developed and instrumentation has improved.

We have examined pmr spectra (at 220 MHz) of cytochromes *c* from nine eukaryotic species (horse, cow, hog, chicken, turkey, duck, pigeon, tuna, and yeast) and three prokaryotic species (*Pseudomonas aeruginosa*, *Desulfovibrio vulgaris*, and *Desulfovibrio gigas*). The purpose of this report is to summarize and extend the descriptive spectroscopy of all regions of the pmr spectra of horse ferri- and ferrocytochromes *c* as a basis for comparison with the spectra of cytochromes *c* from other species and to provide the basis for studying modifications of the cytochrome *c* spectra that occur when the protein interacts with other molecules involved in biological functions.

Experimental Section

The experiments reported here were performed on horse heart cytochrome *c* type III or type VI obtained from Sigma Chemical Co. Spectra acquired from these materials, from these materials subjected to further purification, or from highly purified horse cytochromes *c* from S. N. Vinogradov were equivalent. Typically, solutions containing 15% cytochrome *c* in neutral D_2O (99+%) were examined. Concentrations from 5 to 15% gave equivalent spectra; higher concentrations (~20%) exhibited somewhat broadened resonances. In some cases, the quality (resolution) of the spectra was improved by dissolving dry cytochrome *c* in excess D_2O and evaporating the solvent until the desired protein concentration was obtained. The desired pD value for each solution was obtained by addition of NaOD or DCl. Ascorbic acid was used to reduce ferricytochrome *c* to ferrocytochrome *c;* potassium ferricyanide was used for the reverse oxidation.

Pmr spectra were acquired with a Varian Associates high-resolution spectrometer which operates at a frequency of 220 MHz. Temperature of the samples was regulated to ±1°. Signal-to-noise characteristics of most of the spectra were improved by employing a Varian Associates C-1024 computer of average transients. Resonance positions were measured with respect to the internal sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid with positive shifts to low field from the reference resonance position.

Results and Discussion

General Considerations. The polypeptide chain of horse cytochrome *c* consists of 104 residues in known sequence (Margoliash and Schejter, 1966) and the conformation of native horse ferricytochrome *c* has been determined (Dickerson *et al.*, 1971). A molecule of horse cytochrome *c* contains 875 hydrogen atoms, each of which can, in principal, contribute to the pmr spectrum of the protein. In practice, for the native protein in neutral aqueous solution, several types of proton resonances are difficult or impossible to observe. A detailed inventory of the protons of horse cytochrome *c* is provided in Table I.

The resonance positions for most of these protons can be predicted for certain special conditions (not necessarily achievable experimentally). We term these the primary resonance positions and they are listed in Table I. The special conditions are defined as follows. For CH protons of the side chains of the amino acid residues the polypeptide is dissociated from the influence of the heme and is extended in water at pH 7 and 40° so that the side chains are in a solvent environment. Then protons of a given chemical type will all exhibit resonance at the same position, the primary position, which has been determined from studies of random-coil proteins and model compounds (McDonald and Phillips, 1969). Even for these special conditions, resonance positions of α-CH and peptide NH protons are influenced both by the nature of the residue side chain and the nature of nearest neighbor residues. Thus, at present, we can only indicate a spectral region for the primary resonance positions of these

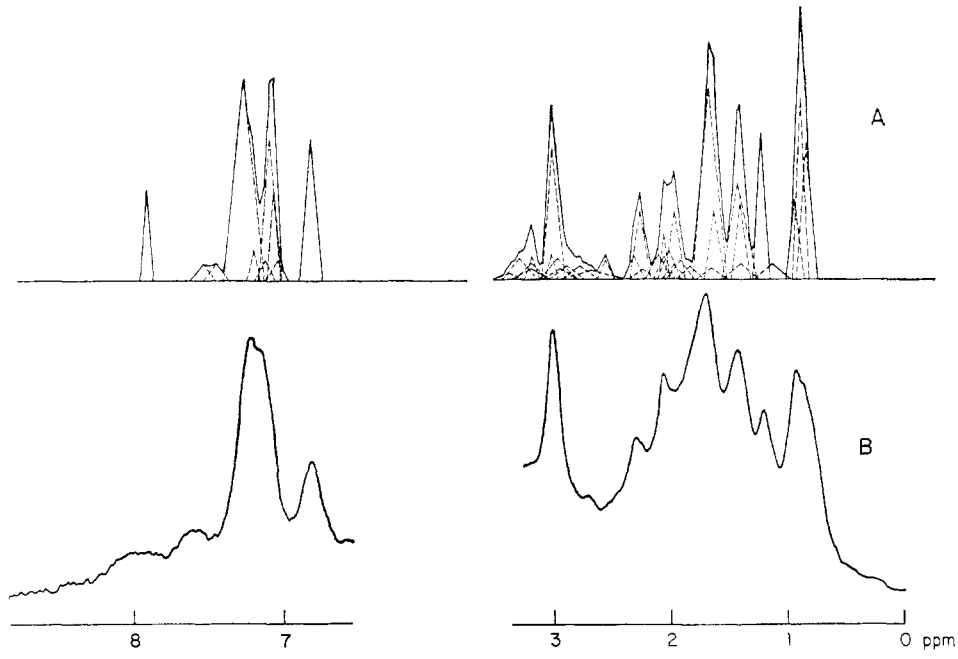

FIGURE 2: (A) Computed primary spectrum of horse cytochrome *c*. (B) Pmr spectrum of horse ferricytochrome *c* in 5 M urea $-D_2O$ (pD 7, 70°).

protons. The primary resonance positions of the heme protons are based on model compound studies of other investigators (Wüthrich, 1970, and references therein) and represent estimates of the resonance positions of a porphyrin moiety dissociated from the polypeptide and having the heme iron in the diamagnetic, low-spin ferrous state. Estimates of the primary positions of NH protons are from studies of model compounds and random-coil proteins in our laboratory (Glickson *et al.*, 1971; Glickson, J. D., and Phillips, W. D., unpublished research).

If cytochrome *c* is dissolved in H_2O, a large central region of the pmr spectrum (~2.7–6.4 ppm) is obscured by the solvent resonance. In D_2O the obscured region, from residual HDO, is smaller (~3.5–5.5 ppm), but all labile protons exchange with deuterons of the solvent and are not observable as protein proton resonances. These exchangeable protons include all NH and OH protons *that have access to the solvent.* In addition, the C-2 proton of histidine exchanges slowly at elevated temperatures. Consequently, in D_2O, the useful pmr spectrum of resonances at or near their primary positions comprises CH resonances of the amino acid side chains, a few heme protons, and NH or OH protons that do not have access to the solvent; the primary resonance positions of all α-CH protons, threonine CH, serine CH_2, and most of the heme protons are in the obscured region. For convenience, we call the spectral region from 0 to 3.5 ppm the aliphatic CH proton region, and the region from 6.4 to 11 ppm the aromatic region. Most of the labile NH proton resonances can be observed if the protein is examined in slightly acidic H_2O (pH <6) at room temperature. In general OH protons and histidine NH protons are "exchange-averaged with the H_2O resonance even in strongly acidic solutions. The primary resonance positions of NH resonances range from about 6.5 to 10 ppm so this region is called the NH absorption region and is largely coincident with the aromatic CH region.

Pmr of Denatured States. If cytochrome *c* is denatured in neutral aqueous solution so that the polypeptide chain is extended in the solvent, we can expect the pmr spectrum to comprise a compilation of resonances which are at or near their primary positions. Consequently, the expected appearance of the spectrum can be computed from the primary resonance positions and the amino acid composition (McDonald and Phillips, 1969). Such a schematic representation for the side-chain CH proton resonances is shown in Figure 2A. Dashed lines represent contributions of individual proton types. Contributions of heme CH protons (a negligible component) have not been included. The amplitude of the aromatic region has been increased by a factor of four compared to the aliphatic region. A similar pmr spectrum (Figure 2B) can be acquired experimentally if ferricytochrome *c* is examined in 5 M urea–D_2O (pD 7) at 70°. The close correspondence of the schematic and actual spectra indicates that the polypeptide chain is indeed extended in solution. All of the NH and the histidine C-2 proton resonances are absent because of exchange. However, the spectrum exhibits broader resonances than would be expected from a true random coil. This broadening may be due to the presence of the heme iron which is likely to be in the high-spin ferric form which causes rapid relaxation of nuclear spin states of nearby protons. It is to be noted that the resonances expected at 10.7 ppm from the porphyrin meso CH protons which are near the heme iron are not observed. Broadening may also arise from a component of aggregated protein undergoing fast exchange with monomeric protein. Resonance widths increase with increased molecular weight (reduced rotational motion) of the observed molecules (McDonald and Phillips, 1970). The schematic representation based on the data in Table I thus serves as a detailed analysis of the spectrum of the denatured protein. That is, the strong composite resonance at 1 ppm can be attributed to methyl groups of leucine, isoleucine, and valine, the resonance at 1.25 ppm to methyl groups of threonine, and so on through the spectrum.

Most solvents other than water that dissolve sufficient amounts of cytochrome *c* for pmr spectroscopy denature the protein. For example, the pmr spectrum of ferricytochrome *c* in trifluoroacetic acid at 41° is shown in Figure 3. The relatively simple spectrum of sharp resonances indicates that the great majority of the resonances are at their primary positions

(primary positions in trifluoroacetic acid are somewhat different than in water (Bak *et al.*, 1968; McDonald and Phillips, 1969)) and demonstrates that the polypeptide chain is extended in solution. In this solvent, the region of α-CH resonance absorption is not obscured and strong NH resonance absorption of lysine amine protons can be observed at 6.8 ppm and of peptide NH from about 7.5 to 8.5 ppm.

Pmr spectra of denatured cytochrome *c* provide three major types of information: (1) confirmation of the validity of primary resonance positions which are a base for comparing shifted resonances in the native protein, (2) identification of the pmr characteristics of the denatured protein so that these can be recognized in the pmr spectrum of the native protein if denaturation occurs during an experiment, and (3) study of the process of denaturation.

The thermal denaturation of ferricytochrome *c* (10% in D_2O, pD 6) was monitored by pmr. At about 55° the spectrum characteristic of the native state (see below) begins to decrease in intensity and a spectrum characteristic of a denatured, extended form of the protein grows in. However, as the temperature is raised or as time progresses at a denaturing temperature, the denatured protein aggregates to a form that is not observable by high-resolution pmr. If the same experiment is performed in 5 M urea (D_2O), aggregation does not occur and the pmr spectrum of the thermally denatured protein can be examined (Figure 2B). Thermal denaturation in 5 M urea begins at 52° and is complete at 62°. The pmr spectrum of the denatured protein increases through this temperature range. The protein renatures reversibly if the temperature is decreased. Since spectra of the protein in the native and denatured states can be observed simultaneously during denaturation, the lifetimes of the states are greater than 10 msec, and since the spectra do not change with time (minutes) at a given temperature intermediate in the thermal denaturation range, the system is in equilibrium. There is some evidence for one or more intermediate states of appreciable concentration between the native and fully denatured states in that the intensities of the resolved resonances of native ferricytochrome *c* do not appear to decrease in intensity in equal degree as denaturation progresses. As noted previously by others (Margoliash and Schejter, 1966), ferrocytochrome *c* is more stable than ferricytochrome *c;* thermal denaturation of ferrocytochrome *c* (in 5 M urea) begins about 70° and is complete at 80°.

Pmr of Native States. The pmr spectra of horse ferricytochrome *c* and ferrocytochrome *c* are quite different from each other and from the spectrum of the denatured protein. Factors that cause resonance positions of protons in native proteins to be shifted from their primary positions have been discussed in detail elsewhere (McDonald and Phillips, 1970; Wüthrich, 1970; Sternlicht and Wilson, 1967). In brief, for diamagnetic ferrocytochrome *c* the most important factors are thought to be ring-current fields, new bonding situations (*e.g.*, hydrogen bonds or coordination of ligands to the heme iron), "solvent" shifts that result from a residue moving from an aqueous environment to a hydrocarbon-like environment in the interior of the protein, and shifts caused by a proton coming close to a charged group of another residue. Ring-current fields of aromatic residues and of much greater magnitude from the porphyrin ring cause resonances of protons close to the faces of the aromatic structures to be shifted to high field; resonances of protons around the periphery of the aromatic rings are shifted to low field. These shifts can be quite large, ranging up to nearly 7 ppm for protons of ligands of the heme iron which are very close to the porphyrin face.

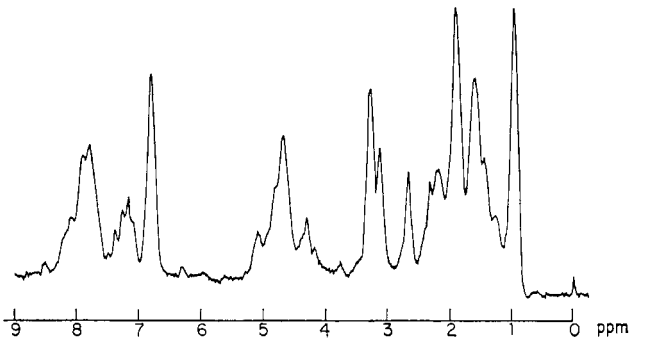

FIGURE 3: Pmr spectrum of horse ferricytochrome *c* in trifluoroacetic acid at 41°.

New bonding situations probably affect few CH resonances except those of the amino acid residues liganded to the heme iron and residues that participate in hydrogen bonds. "Solvent" shifts of methyl resonances appear to be about 0.2 ppm to low field for lysozyme (McDonald, C. C., and Phillips, W. D., manuscript in preparation) and may be assumed to be similar for other proteins; little information is available on "solvent" shifts of other protein protons. Most of the charged groups of cytochrome *c* are on the exterior of the protein and are probably a minor factor in shifting protein proton resonances from their primary positions.

All these factors pertain to native ferricytochrome *c* also. In addition, hyperfine contact interactions and pseudocontact interactions between certain protons and the paramagnetic heme iron can cause very large shifts. Hyperfine contact interactions result from delocalization of spin density from the iron atom through the bonds of the ligands. Such delocalization proceeds readily through π bonds but attenuates rapidly in σ-bond systems. Consequently, large hyperfine contact shifts may occur for the 4 meso CH protons of the porphyrin ring, the 4 porphyrin ring methyl groups, 2 CH protons of the thioether bridges, 4 β-methylene protons of the propionic acid substituents, β protons and imidazole protons of His-18, and the γ protons and methyl group of Met-80, a total of 5 methyl resonances (3 equivalent CH protons each), 16 single CH resonances, and 1 NH resonance (see Figure 1). As we noted earlier, a number of these resonances have been resolved and identified that are well outside the primary resonance region. Several have been observed but not assigned. Some have not been accounted for and presumably are not shifted out of the strongly overlapped central resonance region. A number of other protons may experience smaller hyperfine contact shifts: two methyl groups of the thioether bridges, the two α-methylene groups of the propionic acid substituents, the α proton of His-18, and the β protons of Met-80.

Pseudocontact interactions arise from through-space dipolar interactions between nuclear dipoles and anisotropic electron-spin dipole moment of the heme iron. These interactions may affect resonance positions of protons near the heme iron whether or not they are directly bonded to the prosthetic group. The magnitudes of these interactions decrease with the inverse third power of distance from the iron to a proton and also depend on the angular relationship of this distance vector to the ligand field axis. Moreover, the magnitudes and directions of the pseudocontact shifts depend on the *g* tensor of the electron spin moment. This latter factor is not known for native ferricytochrome *c* at room temperature so it is difficult to evaluate the probable influence of

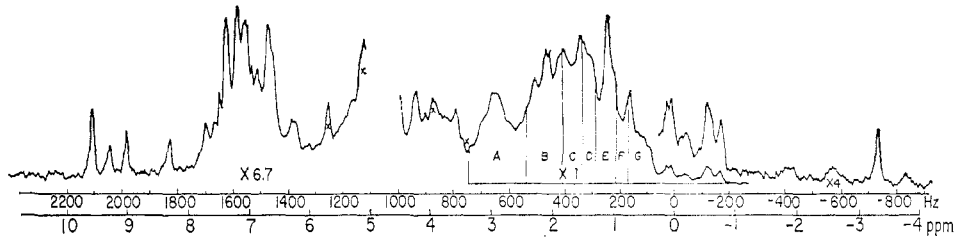

FIGURE 4: Regions of the pmr spectrum of native horse ferrocytochrome *c* in D_2O (pD 6.4, 40°).

pseudocontact shifts on the pmr spectrum of ferricytochrome *c*. Wüthrich has summarized evidence that indicates that pseudocontact shifts are much smaller than the major hyperfine contact shifts for ferricytochrome *c* (Wüthrich, 1970) and indeed the resonances exhibiting the largest contact shifts are firmly assigned to methyl resonances of the Met-80 methyl and heme ring methyl group that are expected to show large hyperfine contact shifts. On the other hand cytochrome *c* exhibits substantial *g*-value anisotropy at 4°K (Salmeen and Palmer, 1968), and it has been estimated that pseudocontact shifts of some of the protons of ferricytochrome *c* may be as great as 6 ppm (Gupta and Redfield, 1970a). From what is known about the anisotropy of the *g* values of cytochrome *c* and other low-spin ferric hemes (Wüthrich, 1970; Gupta and Redfield, 1970a; Redfield and Gupta, 1971; Shulman *et al.*, 1971), it appears that protons near the face of the porphyrin ring should be shifted to low field and protons around the edge of the heme may be shifted to high field (*i.e.*, the pseudocontact shifts are in the opposite directions to ring-current shifts caused by the porphyrin ring).

The positions of proton resonances in the pmr spectrum of a native protein cannot be predicted with accuracy because the environments of the individual protons in the protein molecule are not known in sufficient detail and because quantitative relationships relating resonance shifts with environmental factors are inadequate. Consequently, assignment of resonances and interpretation of the spectra in terms of protein structure must be approached pragmatically. We begin with a consideration of the pmr spectrum of ferrocytochrome *c* since it is not complicated by contact interaction shifts.

Ferrocytochrome c. Regions of the pmr spectrum of horse ferrocytochrome *c* are shown in Figure 4. This spectrum is characteristic of the reduced native protein and, as will be discussed below, is almost invariant over a wide range of temperature and pD.

An expansion of the field region from 0.3 to −4.0 ppm at two temperatures is shown in Figure 5. Four completely resolved resonances are observed between −1 and −4 ppm: three single proton resonances at −1.87, −2.59, and −3.77 ppm and a methyl resonance (three equivalent CH protons) at −3.30 ppm. These have been assigned (McDonald *et al.*, 1969; Gupta and Redfield, 1970a) to the methyl group and to three of the four β- and γ-methylene protons of Met-80. A space-filling (CPK) model of horse ferricytochrome *c* based on atomic coordinates kindly provided by R. E. Dickerson indicates the following relationships with the porphyrin structure: the methyl group lies on ring 1, one of the γ protons lies on the β-meso carbon atom, the other γ proton is near the porphyrin axis and about 2 Å from the ring face, one of the β protons lies against ring 3, and the other lies along the porphyrin axis nearly 3 Å from the ring face. It seems likely that these relationships are similar for the reduced protein but they may not be identical since X-ray diffraction data (Takano *et al.*, 1971) indicates that residues 79 and 81 have somewhat different coordinates in the oxidized and reduced states of the protein. In any case, it is clear that four β and γ protons are all close to the face of the porphyrin moiety but exhibit rather different ring-current shifts. The ring-current field is not expected to be uniform over the porphyrin structure but to be a complex composite of ring currents from the component rings. The resonance at −3.77 ppm must arise from a γ proton since it has been related by cross-saturation (Redfield and Gupta, 1971) with a Met-80 γ-proton resonance of ferricytochrome *c*. It is assigned to the γ proton that lies against the porphyrin face. The resonance at −2.59 ppm is assigned to the β proton lying on ring 3 and the resonance at −1.87 ppm to the γ proton 2 Å from the porphyrin face. The Met-80 methyl resonance may experience low-field ring-current shifts from Tyr-67 and from Phe-82 (alleged to be close to the porphyrin ring in the reduced protein (Takano *et al.*, 1971)) as well as a very large high-field shift from the porphyrin ring.

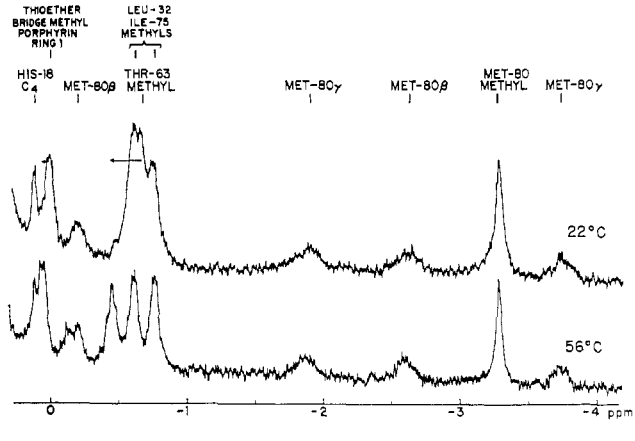

FIGURE 5: High-field pmr spectra of ferrocytochrome *c* at 22 and 56°.

Six partially resolved resonances occur between 0.3 and −1.0 ppm that may serve as useful probes of cytochrome *c* structure. They appear to comprise four methyl resonances at 0.06, −0.45, −0.60, and −0.76, a broad single-proton resonance at −0.17 ppm, and a sharp single-proton resonance at 0.127 ppm (resonance positions at 56°). These resonances may be shifted from their primary positions by ring-current fields of the porphyrin ring or the aromatic side chains. The broad single-proton resonance at −0.17 ppm is probably the β proton of Met-80 not accounted for in the previous paragraph. Few single CH protons in the protein are expected to give resonances as sharp as that at 0.13 ppm since most are broadened by unresolved spin–spin structure. Only glycine α protons (for certain conditions), tryptophan C-2 protons, and histidine C-2 and C-4 protons are not subject to such broadening. Of such protons in ferrocytochrome *c* only the C-2 and C-4 protons of His-18 (both are very close to the face of the porphyrin ring and near the porphyrin axis) are in a sufficiently strong ring-current field to be shifted to 0.13 ppm.

Since the primary position of the C-4 proton is to high field from that of the C-2 proton, we tentatively assign the 0.13-ppm resonance to the C-4 proton of His-18. This represents a shift of 6.84 ppm from the primary position as compared to the shift of the high-field γ proton of Met-80 of 6.36 ppm.

Next, we consider the four methyl resonances that are observed between 0.3 and −1.0 ppm. We have noted that they are common to the pmr spectra of the ferrocytochromes of horse, cow, chicken, duck, pigeon, and tuna and must arise therefore from methyl groups that are common to these proteins. Methyl groups that are shifted considerably to high field from their primary positions by ring-current fields can be affirmed when the atomic coordinates of ferrocytochrome *c* become available to indicate the exact relationship of methyl groups to aromatic structures. Meanwhile, some tentative conclusions can be drawn from the structure of ferricytochrome *c* and a description of the conformational change that occurs when the protein is reduced (Takano *et al.*, 1971). Five methyl groups appear to be situated so that they are subject to strong high-field shifts: one methyl (032CD2 in Dickerson's coordinate nomenclature) of Leu-32 that lies on porphyrin ring 4 and is also in the ring-current field of His-18, one methyl of Ile-75 (075CG1) in the ring-current field of Tyr-67, the methyl of Thr-63 in the ring-current field of Tyr-74, the thioether bridge methyl of porphyrin ring 1 (0001CB) in the ring-current field of Phe-82 (and perhaps of porphyrin ring 1), and the methyl substituent of ring 4 (0004CM) in the ring-current fields of Trp-59 and Tyr-67. The porphyrin ring-methyl resonances of ferrocytochrome *c* have been located at 2.1, 3.4, 3.5, and 3.8 ppm and the thioether bridge methyls at 0.3 and 1.4 ppm (Redfield and Gupta, 1971). These authors assigned the resonance at 2.1 ppm to the methyl substituent of ring 4 because of its high-field shift relative to the other methyl substituents (from the ring-current fields of Trp-59 and Tyr-67). They place the highest field thioether bridge methyl resonance at 0.3 ppm. Our spectra show a minimum at this position and we tentatively assign the resonance at 0.06 ppm to the thioether bridge methyl of porphyrin ring 1. This resonance persists at 80° (for the protein in 5 M urea) after the protein has denatured to an extended form so that all the other high-field methyl resonances have disappeared. At this point it has shifted downfield to 0.27 ppm. It seems probable that the ring-current field of Phe-82 has been removed and the position at 0.27 ppm results from the ring-current field of the porphyrin ring. The resonances at −0.45, −0.60, and −0.76 ppm are assigned to the methyl groups of Leu-32, Ile-75, and Thr-63. The resonance at −0.45 ppm moves to high field as temperature is decreased (Wüthrich, 1970) and also shifts with changes in pH (Gupta and Koenig, 1971) or on the addition of ethylene glycol to the protein solution whereas the resonance positions of all the other resolved high-field resonances remain constant (the 0.06-ppm resonance exhibits a small temperature dependence). The methyl groups of Leu-32 and Ile-75 are buried in the interior of the protein and do not appear likely to change conformation readily with respect to the aromatic structures that cause high-field shifts of their resonances. On the other hand, Thr-63 and Tyr-74 are on the surface of the protein and may be bridged by a hydrogen bond. It seems quite plausible that the geometric relationship of these two residues may be readily modified by temperature or solvent changes with concomitant changes in the ring-current shift of the Thr-63 methyl group. The residues (Leu-32, Ile-75, Thr-63, His-18, Tyr-67, Tyr-74, and Phe-82) alleged to be responsible for the location of the four methyl resonances from 0.3 to −1.0 ppm are present in all the species of ferrocytochrome *c* referred to above that exhibit essentially identical pmr spectra in this spectral region. These residues are also all present in cytochrome *c* from *Candida crusei* (yeast), but in the spectrum of the reduced form of this protein we observe methyl resonances at −0.26, −0.36, −0.74, and −1.14 ppm. That is, the positions of methyl resonances in this field region are quite different from horse and horse-like cytochromes *c* and we conclude that there are substantial differences in conformation between the reduced forms of the horse and *Candida crusei* proteins.

TABLE II: Spectral Distribution of Proton Resonances among the Zones of the Aliphatic Region.

Region (Limits (ppm))	No. of Protons Contributing to Each Zone: Primary Spectrum	Native Ferrocytochrome *c*	Native Ferricytochrome *c*
A (3.50–2.44)	102	102 (0)	112 (10)
B (2.44–1.84)	93	109 (16)	100 (7)
C (1.84–1.53)	98	69 (−29)	66 (−32)
D (1.53–1.31)	62	48 (−14)	39 (−23)
E (1.31–0.98)	36	65 (29)	52 (16)
F (0.98–0.77)	90	25 (−65)	28 (−62)
G (0.77 to −1.00)	0	57 (57)	69 (69)
H (−1.00 to high field)	0	6 (6)	15 (15)

Continuing to low field, we consider the remainder of the aliphatic region from 0.3 to 3.50 ppm. Pmr absorption in this field region comprises overlapped resonances of nearly 500 protons. While individual resonances cannot be distinguished, the resolution of regions of strong absorption is considerably better than in earlier studies at lower frequencies. Some information can be derived from the characteristics of the resonance envelope. First, it is clear that many resonances have been shifted from their primary positions because the residues are in unique protein environments rather than in the solvent. A detailed analysis of this spectral region of the pmr spectrum of hen egg-white lysozyme has shown that such shifts can be accounted for in a semiquantitative fashion by considering only high- and low-field ring-current shifts and "solvent" shifts (McDonald, C. C., and Phillips, W. D., manuscript in preparation). However, such an analysis requires a knowledge of the precise atomic coordinates of all the protein residues; these are not yet available for ferrocytochrome *c*. Meanwhile, a more qualitative analysis can be considered.

First, we divide the primary spectrum (Figure 2A) into zones A–F that correspond to rather discrete regions of resonance absorption. The limits of these regions are shown in Table II. The number of heme and polypeptide protons expected from Table I to contribute to resonance in each zone in the primary spectrum is listed in column 2 of Table II. The native spectrum of ferrocytochrome *c* is divided into the same zones (Figure 4) and zones G and H are added as indicated in Table II. In the primary spectrum, 481 protons contribute to the spectrum to high field from 3.50 ppm. We assume that the total integral of resonance absorption to high field from 3.50 ppm in the native spectrum also arises from 481 protons (*i.e.*, that the net shifting of protons into and out of lower field regions is negligible for the purposes of this discussion). Then the number of protons contributing to each zone in the native

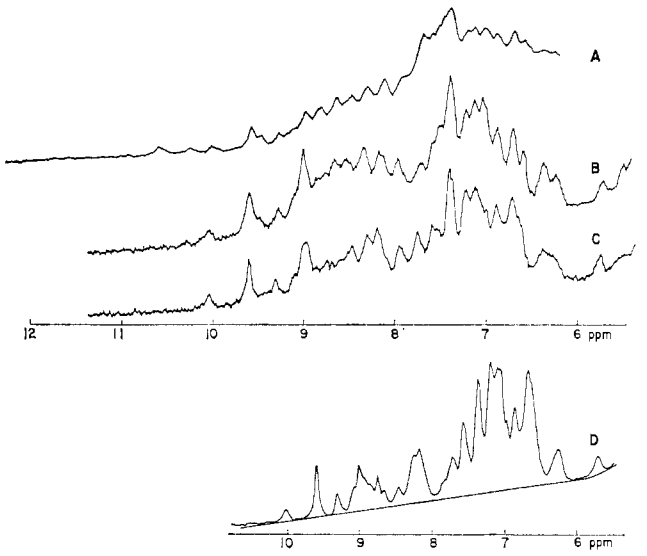

FIGURE 6: Exchange of ferrocytochrome *c* NH protons: (A) protein in H_2O (pH 5, 22°); (B) in D_2O 1 hr (pD 5.0, 22°), amplification 2 × that in A; (C) in D_2O 1 hr at 40°; (D) after heating in D_2O at 55° for 30 min at pD 8.7 (examined at pD 5, 40°).

spectrum can be computed from the ratio of the area of each zone to the total resonance intensity above 3.50 ppm. These numbers are provided in column 3 and the net changes (native minus primary) are indicated in parentheses.

Zone F which comprises 90 proton resonances of 30 methyl groups of leucine, isoleucine, and valine in the primary spectrum has decreased to only 26 proton resonances in the native ferrocytochrome *c* spectrum. It is clear that much of the increase in intensity of native zone G arises from methyl resonances of primary zone F that are subject to high-field ring-current shifts. We have already discussed six resonances (14 protons) that occur from 0.3 to −1.0 ppm in zone G. Most of the buildup in intensity in zone G occurs in the region from 0.30 to 0.77 ppm and arises from rather small shifts of the resonances from primary zone F, *i.e.*, few residues except those discussed earlier appear to be located in the native protein such that they experience sufficiently strong ring currents to shift their resonances into native zone G from primary zones to low field from primary zone F. The remaining loss of intensity of native zone F is accounted for by leucine, isoleucine, and valine methyl groups that are subject to low-field ring-current shifts and solvent shifts. These are expected to be about 0.25 ppm or less and account for some of the increase in intensity of native zone E. Native zone E also includes isoleucine methylene resonances and threonine methyl resonances that are not shifted much from their primary positions (six of the threonine methyls are exposed at the protein surface and unaffected by ring currents) and alanine methyl resonances that are subject to moderate high-field ring-current shifts (*e.g.*, Ala-15). The major components of primary zones A–D arise from hydrophilic residues (*e.g.*, lysine and glutamic acid) that are on the exterior of the native protein. Consequently, most of their resonances are not expected to be shifted much from their primary positions, and we see from Table II that the intensities of these zones are similar in the primary and native spectra although small perturbations cause the overall appearance of the resonance envelopes to be somewhat different. Such perturbations may occur, for example, if only part of a residue side chain is in solvent and we may expect methylene protons near the polypeptide backbone (*e.g.*, β-CH_2) to be in a semihydrophobic environment and subject to solvent shifts. There is a net shift of intensity from primary zones C and D into native zone B which probably arises to a considerable extent from "solvent" shifts of leucine and alanine protons. In summary, the distribution of resonance intensities in the various zones of the native spectrum of ferrocytochrome *c* is qualitatively in agreement with expectation.

We next consider the pmr spectrum of ferrocytochrome *c* from 6 to 11 ppm, *i.e.*, the aromatic CH and the NH spectral regions. If the protein is dissolved in H_2O at pH 5, reduced, and examined, spectrum A of Figure 6 is obtained. Comparison of the intensity from 6 to 11 ppm with that of the aliphatic resonance region indicates that virtually all the 203 labile protons and 51 CH protons with primary positions from 6.0 to 10.5 ppm contribute to the absorption shown in Figure 6A. Most of the resonances are strongly overlapped but a few are resolved at the low-field extreme, *e.g.*, single proton resonances at 10.00, 10.25, and 10.58 ppm and two equivalent protons at 9.60 ppm which have been identified with meso CH protons of the porphyrin ring.

If this reduced protein is lyophilized, dissolved in D_2O (pD 5.0), and examined after 1 hr at room temperature, the spectrum shown as B in Figure 6 is obtained (the amplification of B is about two times that of A). Now, intensity measurements indicate that the observed resonance absorption arises from about 42 unexchanged NH or OH protons as well as from CH protons. In short, about 161 labile protons have exchanged rapidly with the solvent and their resonances are no longer observable but about 42 labile protons appear to be protected from exchange. Consideration of the tertiary structure of cytochrome *c* leads to the conclusion that almost all the labile protons on the amino acid side chains have free access to the solvent and should exchange readily—only about four are in interior locations. However, about 49 of the peptide NH protons appear to be in interior positions so that they are not exposed to solvent. We conclude that most of the unexchanged labile protons arise from such peptide NH protons. It appears that at pD 5.0 and 23° the protein very seldom unfolds to provide access of the solvent to these protons. Even after the protein solution is heated to 40° for 1 hr and then examined at 40° (spectrum C of Figure 6), resonances of about 30 labile protons remain, indicating the conformational stability of the protein. If the protein solution is adjusted to pD 8.7 and held at 55° for 30 min and then examined at pD 5 and 40°, spectrum D of Figure 6 is obtained (spectra A, B, and C are single scan spectra whereas D is a computer-averaged spectrum of 20 scans). Now, the resonance intensity not accounted for by CH protons is reduced to 11 protons. If ferricytochrome *c* is subjected to this heating treatment in moderately basic solution, all the labile protons are exchanged. If the reduced form of this completely exchanged protein is then examined, a spectrum comprising only CH resonances (see Figure 7) is obtained. Comparison of the spectrum of Figure 6D with the spectrum of the completely exchanged ferrocytochrome *c* shows that the unexchanged labile proton resonances in the former spectrum occur between 7.78 and 10.2 ppm and provide all the resonance absorption arising in this region except that from five CH protons (assigned below). The ferricytochrome *c* solution that produced spectrum D was held at room temperature for 7 days and then examined at 40°. The resonance at 10 ppm had disappeared but the remainder of the spectrum was unchanged, showing that the exchange time for the remaining ten or so labile protons is very long at pD 5 and room temperature. It appears that these protons are situated in a very stable region

of the protein tertiary structure that does not unfold (for the conditions tested) to provide solvent access. No obvious assignment is apparent for these protons but they are most likely peptide NH protons in a strongly hydrogen-bonded segment of the molecule. As regards assignment of other resolved labile proton resonances, the extreme low-field positions of the resonances at 10.00, 10.25, and 10.58 ppm in spectrum A suggest that they arise from histidine NH or tryptophan NH resonances. The NH resonance of His-18 should be shifted far to high field by the porphyrin ring-current field. The resonances at 10.25 and 10.58 ppm are tentatively assigned to NH resonances of His-26 and His-33 because these protons exchange readily. Tryptophan NH resonances exchange less readily (Glickson *et al.*, 1971) and so the resonance at 10.00 ppm probably arises from Trp-59.

Considering the CH resonances in the 6.0–10.0-ppm field region (see Figure 7), four are readily assigned from their extreme low-field location and prior work on model compounds (Wüthrich, 1970) to meso protons (α, β, γ, and δ in Figure 1) of the porphyrin ring. Two are coincident at 9.6 ppm and the other two are located at 9.30 and 9.03 ppm. The structure of ferrocytochrome *c* indicates that meso CH's α and δ may be subject to high-field ring-current shifts from Phe-82 and Tyr-67, respectively, and therefore the resonances at 9.03 and 9.30 ppm are tentatively assigned to these protons. The resonance at 9.30 ppm is somewhat broader than the others, perhaps because it is in a somewhat variable ring-current field. Such an effect could be expected if the position of either Tyr-67 or Phe-82 is not rigidly fixed in ferrocytochrome *c*. One might expect Phe-82 at the surface of the molecule and attached to a flexible region of polypeptide chain (Takano *et al.*, 1971) to be more mobile than Tyr-67 which is buried in the protein interior. However, Dickerson and coworkers comment particularly that the location of Tyr-67 is blurred in the electron density map, perhaps indicating a degree of mobility of this residue (Takano *et al.*, 1971). Thus, there exists some evidence favoring assignment of the 9.30-ppm resonance to meso CH δ and the 9.03-ppm resonance to meso CH α.

From Table I we see that the primary aromatic spectrum of cytochrome *c* comprises 47 CH protons. In the spectrum of native ferrocytochrome *c* only 45 resonances are expected in the aromatic region since the C-2 and C-4 resonances of His-18 are shifted far to high field by the ring current of the porphyrin moiety. Of the remaining resonances one is readily assignable to a histidine C-2 proton, by its characteristic shift into the extreme low-field region as pD is decreased (in this case from 7.80 to 8.84 ppm as pD decreases from 7.5 to 4.0). (Compare with a shift from 7.72 to 8.72 for the same proton of free histidine over the same pD range (McDonald and Phillips, 1963).) This resonance exhibits a narrow line width (~10 Hz) when the imidazole ring is in the unprotonated (pD 7.5) or protonated forms (pD 4). As the protein is titrated between these pD values a single exchange-averaged resonance is observed at a position that reflects the ratio of the protonated and unprotonated forms. Midway through the titration (pD ~6) this resonance is broader (~20 Hz), indicating that the lifetime (Pople *et al.*, 1959) of the protonated or unprotonated forms is about 2×10^{-4} sec. Thus, of the two nonliganding histidine residues (His-26 and His-33) it appears that one titrates normally and provides the C-2 resonance that has been described. This resonance is tentatively assigned to His-26 which is on the surface of ferrocytochrome *c* and appears available for interaction with the solvent. His-33 appears from the molecular model also to have access to solvent in the ferri form but the conformation change indicated

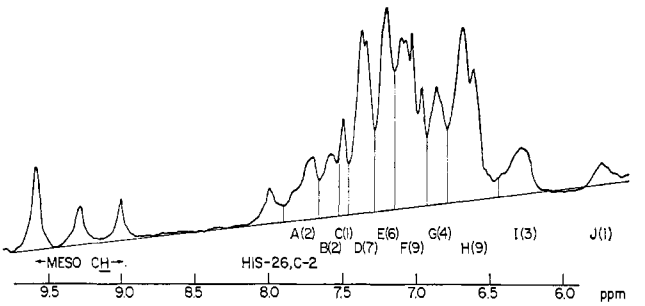

FIGURE 7: Aromatic CH pmr spectrum of ferrocytochrome *c*.

by X-ray studies (Takano *et al.*, 1971) suggests that it may be buried in ferrocytochrome *c* and therefore unavailable for titration. One would expect the C-4 proton resonance of His-26 to shift from about 7.06 to 7.95 ppm as pD is decreased from 4.0 to 7.5. A resonance that probably arises from this proton occurs at 7.62 ppm, pD 4.0–4.5. It shifts to an undetermined position to higher field as pD is increased. The only other resonances in the CH aromatic region that shift appreciably as pD is increased from 4.0 to 10.0 are two sharp resonances that move from 7.60 to 7.52 ppm and 7.10 to 7.04 ppm as pD is increased from 4.0 to 7.5. Only imidazole CH protons of histidine and the C-2 proton of Trp-59 are expected to exhibit resonances with such narrow line widths (~10 Hz); all others are broadened by unresolved spin–spin structure. Thus, we assign these two resonances that move together to the C-2 (7.60 ppm) and C-4 (7.10 ppm) resonances of His-33. The shifts of these resonances with pD are too small to represent any appreciable change in the ionization of the imidazole ring and thus they probably indicate a small conformational change or solvent perturbation of the His-33 environment. The positions of these resonances indicate that only one nitrogen of the imidazole ring of His-33 is protonated.

The only additional narrow resonance observed in the aromatic region is at 6.98 ppm and it is provisionally assigned to the C-2 proton of Trp-59. This resonance is somewhat to high field from the primary position (7.20 ppm) whereas we might have expected a small ring-current shift to low field from the porphyrin ring. However, the position of this resonance is strongly influenced by the nature of the hydrogen bonding of the indole NH proton. This hydrogen bond appears to be to the carboxyl group of the propionic acid substituent of porphyrin ring 4 in ferrocytochrome *c* (Takano *et al.*, 1971) instead of to a water molecule (the hypothetical condition for the primary spectrum).

With regard to the remaining resonances in the aromatic region, it is readily apparent that many resonances have been shifted from their primary positions and that most of these shifts are to high field. This observation is quite compatible with the proposed structure of ferrocytochrome *c* since many aromatic CH protons are in ring-current fields of other aromatic structures so that high-field shifts of their resonances are expected. These relationships are listed in Table III. For convenience of discussion, the aromatic region has been divided into zones A–J in Figure 7. The estimated number of protons (rounded off to integral values) contributing to each zone is indicated in parentheses (derived by assuming total integral intensity in these zones corresponds to 44 protons). A tentative distribution of the aromatic proton resonances among these zones is provided in Table IV. It is based on the indicated ring-current shifts from Table III, the observed intensities in each zone, and consideration of whether the respective pro-

TABLE III: Proposed Ring-Current Shifted Resonances of Aromatic CH Protons.

Residue	Proton Type	Coordinate Designation of Bonded C Atom	Direction of Shift[a]	Source of Ring Current
His-18	C-2	018CD2	Very large H.F.	Porphyrin
	C-4	018CE1	Very large H.F.	Porphyrin
Trp-59	C-2	059CD1	Small L.F.	Porphyrin
	C-4	059CZ1	L.F.	Tyr-67
	C-5	059CH	L.F.	Tyr-67
	C-6	059CZ2	L.F.	Tyr-67
	C-7	059CE2	L.F.	Tyr-67
Phe-10	Ortho	010CD1	H.F.	Tyr-97
	Ortho	010CD2	Small H.F.	Tyr-97
	Meta	010CE1	Small H.F.	Tyr-97
Phe-46	Ortho	046CD1	H.F.	Tyr-48
	Ortho	046CD2	?H.F.	His-26
	Meta	046CE1	H.F.	Tyr-48
			Small L.F.	Porphyrin
	Para	046CZ	Small L.F.	Porphyrin
Phe-82	Ortho		H.F.	Porphyrin
	Meta		H.F.	Porphyrin
	Para		Small H.F.	Porphyrin
Tyr-48	Ortho to OH	048CE2	H.F.	Phe-46
			Small L.F.	Porphyrin
	Meta to OH	048CD2	Small H.F.	Phe-46
			L.F.	Porphyrin
Tyr-67	Ortho to OH	067CE1	Large H.F.	Porphyrin
	Meta to OH	067CD1	Large H.F.	Porphyrin
	Ortho to OH	067CE2	?L.F.	Trp-59
	Meta to OH	067CD2	?L.F.	Trp-59
Tyr-97	Ortho to OH	097CE2	H.F.	Phe-10
	Meta to OH	097CD2	H.F.	Phe-10

[a] H.F., high field; L.F., low field.

TABLE IV: Proposed Distribution of Aromatic CH Resonances among Zones of Absorption.

Zone	Component Resonances: Residue	No. of Protons	Type[a]
A	Trp-59	2	C-4,C-7
B	Trp-59	2	C-5,C-6
C	His-33	1	C-2
D	Phe-10	1	p
	Phe-36	5	All
	Phe-46	1	p
E	His-26	1	C-4
	Phe-10	2	o,m
	Phe-46	1	m
	Phe-82	2	o,m
F	His-33	1	C-4
	Trp-59	1	C-2
	Phe-10	1	m
	Tyr-48	2	m
	Tyr-67	1	m
	Tyr-74	2	m
	Tyr-97	1	m
G	Phe-46	1	o
	Phe-82	1	p
	Tyr-67	1	o
	Tyr-97	1	o
H	Phe-10	1	o
	Phe-46	2	o,m
	Phe-82	2	o,m
	Tyr-48	1	o
	Tyr-74	2	o
	Tyr-97	1	m
I	Tyr-48	1	o
	Tyr-67	1	m
	Tyr-97	1	o
J	Tyr-67	1	o

[a] p, para; o, ortho; m, meta.

tons are in the interior of the protein (causing their resonances to be shifted somewhat to low field) or exposed to solvent on the surface. This analysis will be subject to refinement when more quantitative estimates of the ring-current shifts can be made from the atomic coordinates of ferrocytochrome *c*.

Since the positions of ring-current shifted resonances are strongly dependent on the geometrical relationship between the respective protons and aromatic residues, these resonances are very sensitive to conformational changes of the protein. Such conformational shifts can be observed most readily for resolved resonances but they are also detectable from changes in the envelope of overlapped resonances. Consequently, it is of interest to examine the protein spectra as the environment is changed (temperature, pH, solvent, etc.) to determine whether conformational changes occur.

Ferrocytochrome *c* spectra were examined at 22 and 40° as pD was varied from 7 to 3 and from 7 to 11 at 0.5 pD intervals. The protein dissolved in D_2O had been heated in the oxidized form to exchange NH protons. No change was observed in the overlapped aliphatic spectrum as pD was adjusted from 7 to 4.5. In the high-field region, the resonances tentatively assigned to the thioether bridge methyl of porphyrin ring 1 and the methyl group of Thr-63 shifted a few cycles per second to low field as pD was decreased. In the aromatic region the major changes were the shifts attributed to titration of His-26 that were discussed earlier. Minor shifts attributed to protons of His-33 have also been discussed. Four other aromatic resonances exhibited very small shifts (0.05 ppm or less). Below pD 4.5 the spectra became less well resolved as resonances shifted and broadened. By pH 3 the spectrum was greatly changed. The aromatic and aliphatic regions were less complex, indicating some unfolding of the protein (but not a random-coil spectrum). The porphyrin meso proton resonances disappeared, probably because the heme iron transformed to a high-spin state and increased the relaxation rate of the meso protons so that their resonances became too broad to be detected. The high-field resonances assigned to His-18 and Met-80 disappeared either because these ligands became dissociated from the heme iron or their resonances were severely broadened by the high-spin heme iron. If the pD is again increased from 3 to 4.5, the pmr spectrum becomes more complex again but does not return to the spectrum of the native protein. No change is observed in the spectrum of native ferrocytochrome *c* as pD is increased from 7 to 10. On further increase to pD 11 the resolution of the spectrum becomes much poorer because of resonance broad-

ening but the major features of the spectrum remain the same. In summary, the major elements of the conformation of ferrocytochrome *c* appear to be conserved from pD 4.5 to 10. Except for major shifts of protons of a single histidine residue, only a few minor resonance shifts occur. These probably arise from some changes in conformation of a few residues on the surface of the protein. There is no evidence of any change of the relationship of His-18 to Met-80 to the porphyrin moiety through this pD range.

Ferrocytochrome *c* (10% in D_2O) was examined at about 10° intervals from 56 to 5°. The protein had been preheated in the oxidized state to exchange NH protons. At the beginning of the experiment (56°) the pD was 7.0; at the end it was 6.2. (Such a decrease in pD during the course of a long experiment has been observed repeatedly.) Little change was noted in the overlapped aliphatic region as temperature was changed but some resonances in the high-field and aromatic regions shifted progressively throughout the temperature range studied. In addition the spectra became somewhat less resolved as temperature was lowered indicating a small degree of reversible aggregation at the lower temperatures. The high-field and aromatic regions are compared at 56 and 22° in Figures 5 and 8. The influence of the small pD change is negligible for all resonances except those (indicated as H in Figure 8) of His-26 which titrates in this pD region. The shifts of temperature-dependent resonances are indicated by arrows in these figures. The high-field resonances of the thioether bridge methyl of porphyrin ring 1 and the methyl of Thr-63 shift to low field as temperature is increased, presumably because Phe-82 and Tyr-74 move away from the respective methyl groups. In the aromatic region a number of resonances shift as temperature is changed. All the temperature changes are reversible. There is no sharp division between the aromatic region and α-CH region for ferrocytochrome *c* as resonances are observable in the 5–6-ppm field region. We have rather arbitrarily set the high-field limit of the aromatic region at 5.6 ppm since resonance intensity to lower field appears to account for all the aromatic resonances. It is to be noted, however, that a resonance appeared at 5.5 ppm as the temperature was decreased from 45 to 30°. This resonance of unknown origin disappeared again when temperature was increased through the same range.

In general, from the above temperature study we conclude that the major features of the conformation of ferrocytochrome *c* are conserved from 5 to 56°. However, small shifts of many resonances indicate some progressive small modification of elements of the conformation through the entire temperature interval. There does not appear to be any change in the relationship of the His-18 and Met-80 ligands to the porphyrin moiety.

Similarly, spectra of ferrocytochrome *c* were examined at 40° as ethylene glycol was added to the D_2O solvent (pD 4.5) in stages up to 33%. The spectrum remained constant except for a low-field shift of the resonance which has been tentatively assigned to the methyl group of Thr-63.

Ferricytochrome c. Regions of the pmr spectrum of native horse ferricytochrome *c* (15% in D_2O, pD 5.0, 23°) are shown in Figure 9. This sample was heated to 55° for 30 min at pD 9 to exchange all NH resonances. This treatment does not appear to change the spectrum of CH resonances. Figure 9A shows the aromatic region and neighboring low-field region; Figure 9B shows the aliphatic and neighboring high-field region. Clearly, the characteristics of these spectra are different from those of native ferrocytochrome *c* or of denatured cytochrome *c*. In addition, a number of well-resolved resonances

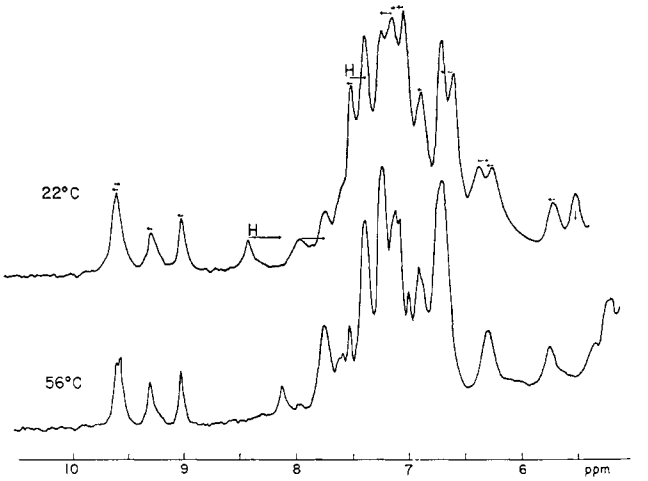

FIGURE 8: Aromatic pmr region at 22 and 56°.

are observed (Figures 9C and D) to low field and to high field from the spectra in Figures 9A and B. These are shifted from the central region because of the paramagnetism of ferricytochrome *c*. Resolved resonances or resolved resonance maxima from overlapped resonances are designated by number in Figure 9. Subscripts refer to the number of protons contributing to each resonance where these numbers have been determined by integration. All resonances with a designated intensity of three protons are believed to arise from methyl groups. The high-field limit of the spectrometer for the operating conditions used in this experiment was about −5000 Hz. Three resonances (70, 71, and 72) are to a high field of −5000 Hz at 23° but move to lower fields as the temperature is increased. Two of these, 70 and 71, were observed at 55° and are shown in the inset of Figure 9D. Certain artifacts (the shaded regions) appear in the spectra of Figures 9C and D because the sample is irradiated with a frequency of 220 MHz modulated by 100 kc. The spectra are acquired in the field region corresponding to resonance absorption of the first high-field side band (*i.e.*, 220.1 MHz). The negative-going resonances 69, 70, 71, and 72 in Figure 9C arise from very high-field resonances of the center-band (220.0 MHz) spectrum. Thus, one has a method of observing those resonances that are beyond the high-field scanning limit of the normal spectrum. The minor resonances, 1 and 2, in Figure 9D arise from very low-field resonances of the second high-field sideband (220.2 MHz) spectrum.

The positions of resonances 1–78 are shown as a function of the inverse absolute temperature in Figures 10–12. The resonances of Figure 12 are clearly in field regions such that the corresponding protons must be experiencing interaction with the paramagnetic heme iron. All these resonances are temperature dependent. Many of the resonances in the normal spectral region (−2 to 10 ppm) also show a dependence on temperature (Figures 10 and 11) but many others, particularly the major resonance maxima comprising many coincident resonances, are essentially unaffected by temperature. These temperature effects may arise from a temperature-dependent conformational change or from expected temperature dependences of contact shifts (or both). Since most of the major resonance maxima are temperature independent, we favor the view that the effects of temperature on conformation are small (*i.e.*, the general conformation of the polypeptide chain remains constant) and, consequently, the resonances showing temperature shifts in the normal spectral region are those arising from protons subject to relatively small contact inter-

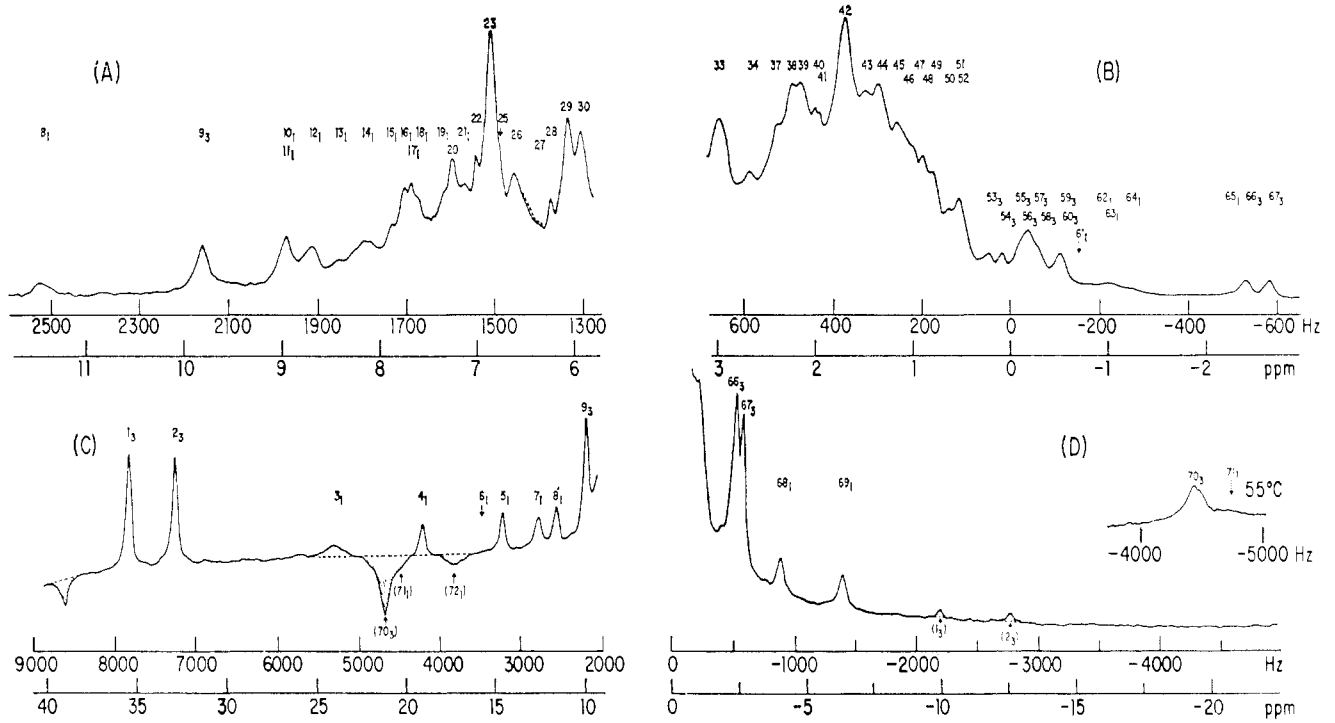

FIGURE 9: Regions of the pmr spectrum of native horse ferricytochrome *c* in D_2O (pD 5.0, 23°): (A) aromatic region; (B) aliphatic and near high-field region; (C) low-field contact-shifted resonances; (D) high-field contact-shifted resonances.

actions. If the resonances showing temperature-dependent positions are all (or mostly) subject to contact shifts, clearly, as will be documented below, many of them must be shifted by pseudocontact interactions since the total number of temperature-dependent resonances is much larger than the number of resonances expected to be subject to contact hyperfine interaction. This conclusion by itself is not in contradiction with earlier proposals (Wüthrich, 1970) that most of the pseudocontact shifts are relatively small compared to the major hyperfine contact shifts since most of the temperature-dependent resonances remain within the limits of the normal spectral region. However, if we observe many small pseudocontact interactions, we may expect the relatively small number of protons close to the heme iron to show much larger pseudocontact shifts. These are in many cases also the protons expected to be subject to large hyperfine contact interactions. Consequently, we agree with the conclusion (Gupta and Redfield, 1970a) that although the contact shifts of the resonances outside the normal spectral region probably arise primarily from hyperfine contact interactions they may also contain a sizable pseudocontact component which may for individual protons reinforce or oppose the direction of the hyperfine shifts.

Both hyperfine and pseudocontact shifts normally exhibit a linear dependence on the inverse of the absolute temperature (Curie law). Contact-shifted resonances of ferricytochrome *c* exhibit a variety of types of temperature dependences: Curie law (*e.g.*, resonances 1, 2, 4, and 5), nonlinear decreasing shift with increasing temperature (*e.g.*, 3, 7, 65, and 70), almost no temperature dependence (8), and nonlinear increasing shift with increasing temperature (9 and possibly 25). The reason for this unusual temperature behavior is not yet known. Some of the factors that may cause temperature dependence of contact-shifted resonances to depart from Curie law behavior have been introduced (Kurland and McGarvey, 1970). Neither these factors nor a temperature-dependent conformational change seems adequate to explain the observed behavior. Temperature dependences of turkey ferricytochrome *c* contact-shifted resonances have been provided (Wüthrich, 1970). They were not extended to sufficiently high temperatures to exhibit the nonlinear temperature dependences reported here for horse ferricytochrome *c*.

The dependences of the positions of the numbered resonances on pD were examined for the pD range from 4 to 9 (ferricytochrome *c* 15% in D_2O, 23°, preexchanged). The spectra were almost invariant from pD 9 to 5 indicating a constant conformation of the protein polypeptide chain through this pD range. There were, however, shifts of 50–100 Hz of some of the resonances that show large contact shifts (*e.g.*, 1, 2, 3, 7, 9, 68, and 70). As pD is decreased from 5 to 4.5, many resonances begin to shift suggesting a general conformational change. Between pD 4.5 and 3.0 the low-field and high-field contact-shift resonances decrease in intensity and disappear (Gupta and Koenig, 1971) and many changes occur in the central-field region. Spectra at these pD values are compared in Figure 13. The aromatic region becomes more like the spectrum of the denatured protein (note additional histidine C-2 resonance at X) but the aliphatic spectrum, although changed, indicates that the protein retains some definite tertiary conformation. In Figure 13 positions of temperature-dependent resonances at pD 5 are indicated by arrows. These are presumed to be contact-shifted resonances. It is clear that there is appreciable loss in intensity on going from pD 4.5 to 3.0 and that the intensity losses correlate well with positions of contact-shifted resonances. No further change occurs on decreasing pD from 3 to 2. We concur with Gupta and Koenig that as pD is decreased from 4.5 the conversion of the heme iron to the Fe^{3+} ($S = {}^5/_2$) high-spin state causes most protons undergoing contact interaction to be broadened to the point that they are no longer observable. The residual spectrum arises from protons that are not close to the heme iron and its complexity indicates that some globular conformation is retained. Above pD 9, horse ferricytochrome *c* converts to new forms. Spectra of the extreme contact-shifted resonances of

TABLE V: Assignment of Contact-Shifted Resonances of Horse Ferricytochrome *c*.

Resonance Designation	Field Position (23°, pD 5) (ppm)	Assignment	Ref
1	35.42	Methyl group of porphyrin ring 4	*a–c*
2	32.85	Methyl group of porphyrin ring 2	*a–c*
9	(9.81)	Methyl groups of porphyrin rings 1 and 3	*b–d*
25	(6.77)		
70	−24.20	Methyl group of Met-80	*a,b,e*
71	−25.1	Met-80 γ proton	*d*
72	−28.1	Met-80 γ proton	*c*
66	−2.44	Heme thioether bridge methyl, porphyrin ring 2	*b–d*
67	−2.68	Heme thioether bridge methyl, porphyrin ring 1	*b–d*
4	19.08	β-CH of propionic acid substituent of porphyrin ring 4	*c,d*
5	14.54	β-CH of propionic acid substituent of porphyrin ring 4	*d*
3	24.05	Thioether bridge CH substituent to porphyrin ring 2	*d*
68	(−3.99)	Heme meso CH protons	*c*
69	(−6.33)		

[a] Wüthrich, 1970. [b] Gupta and Redfield, 1970a. [c] Redfield and Gupta, 1971. [d] This work. [e] Wüthrich, 1969.

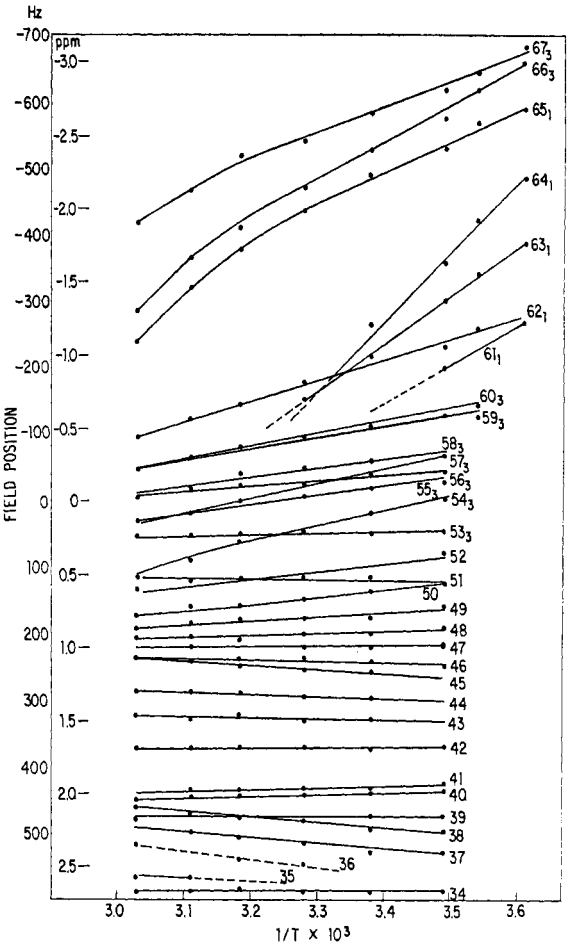

FIGURE 10: Temperature dependence of ferricytochrome *c* resonances.

these forms have been studied (Gupta and Koenig, 1971); it was concluded that the heme iron remains in the low-spin state but Met-80 is no longer a ligand.

Contact-shifted resonances that have been assigned are summarized in Table V. The first two columns give resonance designation and field positions as in Figure 9. The assignment of resonances 9 and 25 to the methyl groups of porphyrin rings 1 and 3 is based on approximate correspondence of these resonances to the positions previously assigned (Gupta and Redfield, 1970a; Redfield and Gupta, 1971) and on the temperature dependences observed in Figure 11. Resonances 71 and 72 are assigned to γ protons of Met-80 because they should exhibit contact shifts similar to the methyl resonance of Met-80. Redfield and Gupta ascribe the shifts of resonances 66 and 67 (their C_1 and C_2) to pseudocontact interactions of the thioether bridge methyls. We have assigned a resonance of ferrocytochrome *c* (see above) to the thioether bridge methyl associated with porphyrin ring 1 that appears to be identical with the methyl group giving rise to resonance 67 by the cross-saturation technique. Resonance 66 therefore is assigned to the thioether bridge methyl associated with porphyrin ring 2. Both methyl groups probably have primary positions about 1.5–2.0 ppm. The thioether bridge methyl associated with ring 1 is thought to be shifted to high field by the ring current of Phe-82 in the reduced protein but not in the oxidized protein. Thus, the contact shifts of resonances 66 and 67 are about 4 ppm. Probably this shift is made up of both high-field hyperfine contact and pseudocontact components. If, as has been proposed (Redfield and Gupta, 1971; Shulman *et al.*, 1971), spin density delocalization is greatest for porphyrin rings 2 and 4 and pseudocontact interactions in the heme plane are greatest for protons associated with porphyrin rings 1 and 3, the hyperfine contribution to the shift of resonance 66 should be larger than for resonance 67, whereas the pseudocontact contribution should be greatest for resonance 67. It is interesting to note that the nonlinearity of the temperature dependence of the resonance position is considerably greater for resonance 66 than resonance 67. Resonance 4 exhibits interspin cross-relaxation with resonance 1 and therefore must be in contact ith the methyl group on porphyrin ring 4 (Redfield and Gupta, 1971). These authors therefore assigned this resonance to a β proton of Leu-32. However, this proton would require a very large low-field pseudocontact shift to place it at 19 ppm. We feel that it is much more reasonable to assign this resonance to a β proton of the propionic acid substituted on porphyrin ring 4 since this proton also appears from the molecular model to be in contact with the methyl group and because it should experience a large low-field hyperfine contact shift similar in magnitude to that of the methyl group (resonance 1). The other β proton of the propionic substituent that is not in contact with the methyl group should exhibit a similar contact shift. This proton is assigned to resonance 5 since it is relatively close to resonance 4 (the contact shifts of resonances 4 and 5 depend on the stereochemical relationship of these protons to the porphyrin ring and are therefore not expected to be identical)

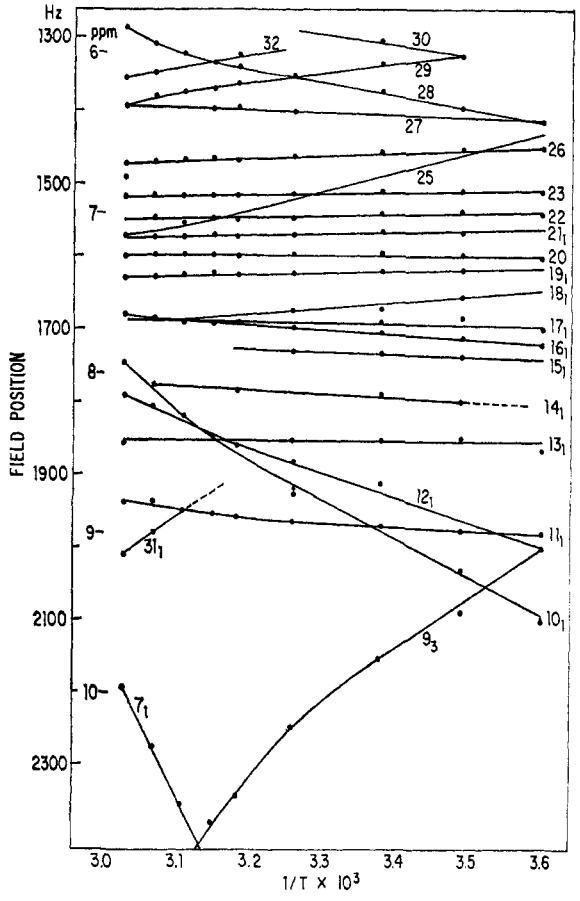

FIGURE 11: Temperature dependence of ferricytochrome *c* resonances.

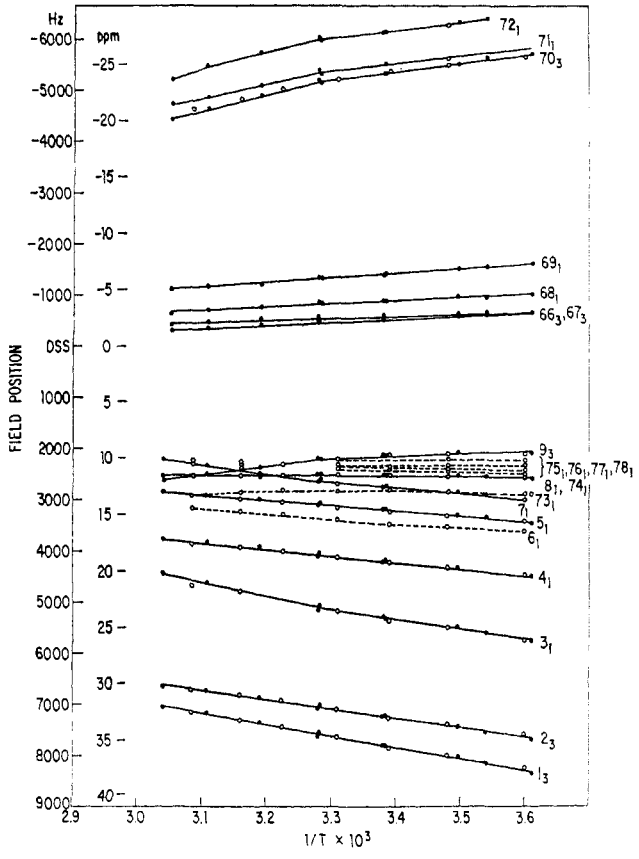

FIGURE 12: Temperature dependence of ferricytochrome *c* resonances: solid lines and filled circles for CH resonances; dashed lines and open circles for exchangeable protons observed in H_2O (pH 5).

and because resonances 4 and 5 show similar temperature dependences (Figure 12). The thioether bridge CH proton associated with porphyrin ring 2 should experience a large low-field hyperfine contact shift similar to the methyl group on this ring (resonance 2). Consequently, we assign resonance 3 to this proton. This resonance is broader than the others and for this reason was assigned previously to His-18 (Redfield and Gupta, 1971). However, it sharpens up if the protein solution is made 1.5% in gelatin, an effect that would not be expected for a His-18 proton. We attribute its usual breadth to the fact that, if our assignment is correct, the proton is on the surface of the protein and may interact with a flexible surface residue or another cytochrome *c* molecule. The heme meso CH protons are expected from model compound studies (Wüthrich, 1970) to experience large hyperfine contact shifts and/or pseudocontact shifts to high field. Accordingly, resonances 68 and 89 are assigned to two of the four meso protons; absolute assignment to particular protons has not yet been made. The other two meso proton resonances are probably in the group of temperature-dependent resonances numbered 61–65. There may be a substantial pseudocontact contribution to the contact shifts of these protons. Since the contact shifts of the methyl groups on porphyrin rings 1 and 3 are much smaller than those on rings 2 and 4, we expect the β protons of the propionic acid substituent on ring 3 and the thioether bridge CH associated with ring 1 to exhibit relatively small low-field contact shifts and to be located in the normal resonance region; resonances 10, 12, and 28, for example, are possible candidates. The only other protons expected to show large low-field contact shifts (see assignments of myoglobin contact-shifted resonances (Shulman *et al.*, 1969)) are the C-2 and C-4 imidazole protons of His-18. However, the remaining resonances, 7 and 8, showing relatively large shifts, are probably too narrow to be assigned to His-18.

As indicated in Figure 12, resonances of 27 protons occur well outside the normal spectral region. If the assignments of Table V are correct, most of these protons are expected to experience large hyperfine contact shifts and their positions can be rationalized on that basis alone (although the shifts of 66 and 67 may be somewhat large for hyperfine contact shifts and resonances 7 and 8 are unassigned). In all, 44 protons listed earlier are expected to exhibit observable contact shifts. Figures 10–12 reveal that at least 74 protons have resonances that shift with temperature. Clearly many resonance positions are subject to effects of pseudocontact interactions or to a temperature-dependent conformational change.

We next consider the distribution of all aliphatic resonances. A spectrum of ferricytochrome *c* obtained at 23° (similar to Figure 9B) was divided into the same zones used for ferrocytochrome *c* (see Table II). Again it was assumed that the total intensity to high field from 3.50 ppm corresponded to 481 protons. The number of protons contributing resonance absorption to each zone calculated by integration is provided in Table II. The conformational change between ferricytochrome *c* and ferrocytochrome *c* indicated by X-ray diffraction undoubtedly causes some resonance positions to be shifted but is unlikely to cause major changes in the protons contributing to the various zones. The differences between ferrocytochrome *c* and ferricytochrome *c* zonal integrals in Table II more likely arise primarily from contact shifts in

the ferricytochrome *c* spectrum. Zone H comprises resonances of Met-80 and heme protons that have been shifted into this region by contact shifts as discussed above. The β protons of Met-80 are probably shifted considerably to low field from their location in the ferrocytochrome *c* spectrum by contact shifts and should not appear in zone H. By and large the differences in the zonal integrals between the oxidized and reduced proteins are quite small (3–13 protons) and relatively small pseudocontact shifts can account for most of these changes. For example, in zone G which contains many methyl resonances, it appears from temperature dependences of their resonances that at least seven methyl groups experience high-field pseudocontact interactions. These are presumably mostly methyl groups around the periphery of the porphyrin ring (*e.g.*, Leu-94) that also experience low-field ring-current shifts. Consequently, in the spectrum of ferrocytochrome *c* they may occur in zone E. Thus, one can rationalize the decrease in zone E and the increase in zone G relative to ferrocytochrome *c*. Similarly, low-field pseudocontact shifts in the region of 2.5 ppm (Figure 10) may account for the small decrease in zone B and corresponding increase in zone A.

If the aromatic and NH regions of the pmr spectrum of horse ferricytochrome in H_2O (pH 5.0, 23°) are examined, one observes a broad band of many overlapped resonances from 6 to 10 ppm. Seven readily exchangeable (presumably NH) single proton resonances have been detected below 10 ppm. Their positions are indicated in Figure 12 by open circles. Resonance 6 has been reported previously (Redfield and Gupta, 1971) and assigned to the His-18 imidazole NH. However, if this were the case, it should exhibit a resonance width similar to resonance 70 because of similar proximity to the heme iron. Since its resonance width is much narrower, comparable to resonances 4, 5, 7, and 8, it more likely arises from a pseudocontact-shifted peptide NH proton farther from the heme iron. Pseudocontact shifts of peptide NH protons along or near the heme axis (*e.g.*, Thr-19) perpendicular to the heme plane should be in the low-field direction. The other exchangeable proton resonances 73, 74, 75, 76, 77, and 78 (74 and 8 are overlapped) presumably also arise from pseudocontact-shifted peptide NH protons. Since the primary positions of peptide NH extend to high field from 9 ppm and since these particular peptide NH protons near the faces of the porphyrin moiety are subject to high-field directing ring-current shifts, the pseudocontact shifts of these protons range up to 7 ppm or more even though (from their line widths) they are not as close to the heme iron as many other protons (*e.g.*, Met-80 and His-18 side chains and heme meso protons). This finding perhaps provides the best evidence that the pseudocontact shifts of some resonances may be of very considerable magnitude. Pseudocontact-shifted, exchangeable proton resonances have also been detected in the low-field spectrum of cyanoferrimyoglobin and accounted for in a similar fashion (Sheard *et al.*, 1970).

If the protein is dissolved in D_2O at 23° (pD 5), all but about ten of the NH protons exchange immediately (<10 min) but these ten persist for some time. If the protein is reduced one finds that these slowly exchanging NH resonances are the same as those that are very resistant to exchange in the reduced state. Therefore, we conclude that most of the polypeptide chain of ferricytochrome *c* has at least occasional access to the solvent at room temperature but that there is a region of rigid structure that does not permit solvent access to about ten NH protons and this is the same region of tertiary structure that is most stable in ferrocytochrome *c*. If the oxidized protein is heated in D_2O for a short time in moderately alkaline solution (*e.g.*, 30 min at pH 9 and 55°), all the NH protons are exchanged.

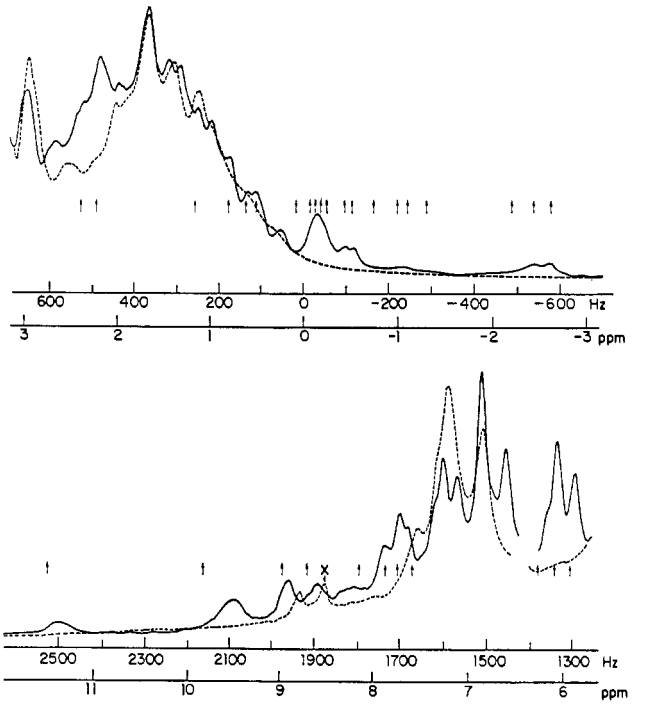

FIGURE 13: Comparison of ferricytochrome *c* spectra at pD 4.5 and 3.0.

A spectrum of the aromatic CH region of ferricytochrome is provided in Figure 9A and temperature dependences of many of these resonances have been indicated in Figure 11. The spectrum is quite different from that of the reduced protein so that it has not been possible to identify corresponding resonances in the oxidized and reduced states and no detailed analysis of this region has been attempted. This may be possible eventually by the cross-saturation technique. Most of the aromatic resonances remain in the 6–8-ppm region and only a few show much temperature dependence. Presumably, these are pseudocontact-shifted resonances of some of the aromatic protons that are close to the porphyrin ring. The main differences then between the spectra of the oxidized and reduced states must be a consequence of differences in the ring-current shifts that affect many of the aromatic CH resonance positions. Relatively small conformation changes can cause quite marked changes in ring-current shifts. The fact that most of the resonances are essentially temperature independent is taken as strong evidence that the major features of the conformation of horse ferricytochrome *c* are not changed on heating until near the thermal denaturation temperature.

Redox Exchange of Heme Meso Protons. Castro and Davis (1969), from model compound studies, have proposed that oxidation and reduction of heme proteins such as cytochrome *c* involve protonation of the meso position and result in proton exchange. Since the meso CH protons of ferrocytochrome *c* are readily observable and firmly assigned, the hypothesis was put to experimental test.

Ferricytochrome *c* was preexchanged in D_2O to remove NH resonances from the spectral region of the heme meso CH protons. This procedure does not exchange the meso protons. The protein was reduced in D_2O (pD 7, 23°) with ascorbic acid (preexchanged in D_2O) and the pmr spectrum of the meso CH region was examined. The protein was subjected to

two further cycles of oxidation with ferricyanide and reduction with ascorbic acid and examined after each reduction. No reduction in intensity of any of the meso CH resonances was observed. We conclude that reduction and oxidation of cytochrome *c* for the conditions described here do not involve protonation or proton exchange at any of the heme meso positions.

Acknowledgment

We are indebted to the following people for assistance in this research: Messers. F. V. Ferrari, F. W. Barney, and L. J. Rizzardi for technical assistance; Dr. S. N. Vinogradov, Wayne State University, for a gift of purified horse cytochrome *c;* Professor R. E. Dickerson, California Institute of Technology, for provisional coordinates of horse ferricytochrome *c*, used with the understanding that they are subject to further refinement.

References

Bak, B., Dambmann, C., Nicolaisen, F., and Pedersen, E. J. (1968), *J. Mol. Spectrosc. 26*, 78.

Castro, C. H., and Davis, H. F. (1969), *J. Amer. Chem. Soc. 91*, 5405.

Chance, B., Estabrook, R. N., and Yonetani, T. (1966), Hemes and Heme Proteins, New York, N. Y., Academic Press.

Dickerson, R. E., and Geis, I. (1969), The Structure and Actions of Proteins, New York, N. Y., Harper and Row.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margoliash, E. (1971), *J. Biol. Chem. 246*, 2511.

Glickson, J. D., Phillips, W. D., and Rupley, J. A. (1971), *J. Amer. Chem. Soc. 93*, 4031.

Gupta, R. K., and Koenig, S. H. (1971), *Biochem. Biophys. Res. Commun. 45*, 1134.

Gupta, R. K., and Redfield, A. G. (1970a), *Science 169*, 1204.

Gupta, R. K., and Redfield, A. G. (1970b), *Biochem. Biophys. Res. Commun. 41*, 273.

Kamen, M. D., Dus, K. M., Flatmark, T., and de Klerk, H. (1971), Electron and Coupled Energy Transfer in Biological Systems, Vol. 1, Part A, New York, N. Y., Marcel Dekker.

Kowalsky, A. (1962), *J. Biol. Chem. 237*, 1807.

Kowalsky, A. (1965), *Biochemistry 4*, 2382.

Krejcarek, G. E., Turner, L., and Dus, K. (1971), *Biochem. Biophys. Res. Commun. 42*, 983.

Kurland, R. J., and McGarvey, B. R. (1970), *J. Magn. Resonance 2*, 286.

Mandel, M. (1965), *J. Biol. Chem. 240*, 1586.

Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.

McDonald, C. C., and Phillips, W. D. (1963), *J. Amer. Chem. Soc. 85*, 3736.

McDonald, C. C., and Phillips, W. D. (1967), *J. Amer. Chem. Soc. 89*, 6332.

McDonald, C. C., and Phillips, W. D. (1968), Third International Conference on Magnetic Resonance in Biology, Warrenton, Va.

McDonald, C. C., and Phillips, W. D. (1969), *J. Amer. Chem. Soc. 91*, 1513.

McDonald, C. C., and Phillips, W. D. (1970), *Biol. Macromol. Ser. 4*, 1.

McDonald, C. C., Phillips, W. D., LeGall, J., and Vinogradov, S. N. (1970), Fourth International Conference on Magnetic Resonance in Biology, Oxford, England.

McDonald, C. C., Phillips, W. D., and Vinogradov, S. N. (1969), *Biochem. Biophys. Res. Commun. 36*, 442.

Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High Resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw-Hill, p 218.

Redfield, A. G., and Gupta, R. J. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 405.

Salmeen, I., and Palmer, G. (1968), *J. Chem. Phys. 48*, 2049.

Sheard, B., Yamane, T., and Shulman, R. G. (1970), *J. Mol. Biol. 53*, 35.

Shulman, R. G., Glarum, S. H., and Karplus, M. (1971), *J. Mol. Biol. 57*, 93.

Shulman, R. G., Wüthrich, K., Yamane, T., Antonini, E., and Brunori, M. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 623.

Sternlicht, H., and Wilson, D. (1967), *Biochemistry 6*, 2881.

Takano, T., Swanson, R., Kallai, O. B., and Dickerson, R. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 397.

Wüthrich, K. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1071.

Wüthrich, K. (1970), *Structure Bonding 8*, 53.

Wüthrich, K. (1971), Probes of Structure and Function of Macromolecules and Membranes, Vol. 2, New York, N. Y., Academic Press.

Wüthrich, K., Aviram, I., and Schejter, A. (1971), *Biochim. Biophys. Acta 253*, 98.