chemistry, New York, Academic, p. 181.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.
Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.
Tonomura, Y., and Morita, F. (1959), *J. Biochem. (Tokyo) 46*, 1367.
Young, D. M., Himmelfarb, S., and Harrington, W. F. (1965), *J. Biol. Chem. 240*, 2428.

Nuclear Magnetic Resonance Studies of Cytochrome *c*. Possible Electron Delocalization*

Arthur Kowalsky†

ABSTRACT: New resonances at abnormally high and low fields have been observed in the proton magnetic resonance spectrum of horse heart ferricytochrome *c*. They are not found in the spectrum of the reduced protein, nor are they found in the spectrum of the oxidized form either in 8 M urea or in acid solution. There are about four protons involved in each low-field resonance. These resonances have been used to estimate the rate of electron exchange between oxidized and reduced cytochrome *c*. Similar absorptions have been found in the spectra of cytochrome *c* of other species, of myoglobin, of the heme peptides, and of the hematohemin of cytochrome *c*. A comparison of the complete nuclear magnetic resonance spectrum of oxidized and reduced heme peptides indicates that the anomalous resonances of these compounds may arise from the methine bridge protons of the porphyrin ring. Other possible partial sources are the ethyl and methylene protons immediately adjacent to the porphyrin ring and the protons α to carboxyl and amino groups. However, it does not seem likely that the contact resonances of the peptides are the same as those of ferricytochrome *c*.

The abnormal resonances may be the result of electron delocalization with consequent hyperfine contact interaction between the electron and certain protons of the heme or of the protein. They may also result from a pseudo-contact interaction. In view of the simplicity of the protein contact spectra, the latter interaction does not seem likely.

The proton magnetic resonance spectra of proteins consist of a series of broad absorptions whose total width covers a range of roughly 10 ppm (Kowalsky, 1962a; Bovey *et al.*, 1959). The shapes of these absorptions, of small molecular weight proteins, are characteristic of the composition of the protein (Jardetzky and Jardetzky, 1957) and also of the internal mobility or flexibility of the peptide chain (Kowalsky, 1962a). In this respect, differences observed between the spectra of the oxidized and reduced forms of cytochrome *c* have been attributed mainly to modifications in the conformation of the protein moiety with subsequent changes in the mobility of portions of the peptide chain. The electronic effect, *i.e.*, the influence of an unpaired electron on the spectra of a previously diamagnetic species, was assumed to be small. Recent experiments in some small organic paramagnetic complexes (Eaton *et al.*, 1962) have pointed up the fact that, in such systems, the electronic effect is far from negligible. Indeed, new resonances, far removed from the normal range, have now been found in the spectrum of oxidized cytochrome *c* which are definitely and intimately related to the presence of the paramagnetic metal ion. This paper is a report of investigations concerned with these new resonances, the conditions of their appearance, their source, and other information which can be derived from measurements on them. These new resonances will be referred to as "contact resonances," and a distinction between the two possible types of contact resonance will be made in the discussion.

Experimental Section

Materials. Cytochrome *c*, type III from horse heart, was obtained from Sigma Chemical Co. Various lot numbers were used. The product usually contained some reduced form. Completely oxidized cytochrome *c* was obtained by treating the commercial material with the minimum amount of potassium ferricyanide. The

* From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. *Received April 24, 1965.* This work was supported by U. S. Public Health grants (AM-06940 and GM-12446). A preliminary report of this paper has been presented (Kowalsky, 1964).

† Research Career Development Awardee, U. S. Public Health Service.

TABLE I: Positions of Contact Resonances in Ferricytochrome *c* and Derivatives.

Compound	Number of Unpaired Electrons	Positions (τ units)
Ferricytochrome *c*, pD 5.4–9.4	1	$+33.9 \pm 0.2$, -21.6 ± 0.1, -24.3 ± 0.1
Heme undecapeptide, pD 10.4	1	-6.1, -8.2, -10.2, -19.3, -22.7
Heme undecapeptide + 8 M urea, pD 10.6		-5.9, -7.5, -18.5, -19.9
Heme octapeptide, pD 8.1	1	-4.6, -9.8, -16.4, -22.3
Hematohemin, pyridine-d_5 solvent	5	-15 to -50
Hematohemin, 20% pyridine-d_5–0.2N NaOD–D_2O solvent	1	None observed

mixture of protein and potassium ferrocyanide was then shaken with a twofold excess of Dowex-1 chloride as determined experimentally. The mixture was centrifuged, and the supernatant was drawn off for further use. Reduced cytochrome *c* was prepared by reduction with ascorbic acid and subsequent crystallization (Hagihara *et al.*, 1958). The protein was either dialyzed or put through a Sephadex G-25 column before it was used. Cytochrome *c* from pigeon breast muscle and from hog kidney was obtained through the courtesy of Dr. Ronald Estabrook. Metmyoglobin was obtained from Mann Research Laboratories, Inc. It was reduced, *in situ*, with a small amount of sodium dithionite.

The heme undecapeptide and heme octapeptide from pepsin-digested cytochrome *c* were prepared according to literature procedures (Tuppy and Paléus, 1955; Harbury and Loach, 1960). Purity was estimated by iron analysis of the samples. They were stored in the lyophilized state at $-20°$ until they were used. These were also reduced *in situ* with a small amount of sodium dithionite. The hematohemin from cytochrome *c* was prepared according to the procedure of Paul (1950) and stored at $-20°$.

D_2O was obtained from the Stuart Oxygen Co. and was distilled before it was used. Pyridine-d_5 was obtained from Merck Sharp and Dohme of Canada, Ltd.

Instrumentation. Spectra were obtained on a Varian HR-60 spectrometer. A Hewlett Packard 200AB audio oscillator and a Hewlett Packard 5210 counter were used in calibrating the frequency scale. Spectra were recorded on a Varian G-10 recorder or, in later phases of the work, on a Moseley 2D-2AM x–y recorder. An additional dc level control was inserted between the spectrometer output and the recorder. The high sensitivity insert V-4331-HS was used. The preamplifier was modified to achieve a lower noise figure and the output filter of the spectrometer was also modified to achieve more efficient filtering.

Procedure. Aqueous samples were observed, with one exception, in D_2O solution. The hematohemin derived from cytochrome *c* was observed in pyridine-d_5. The procedure for the preparation of samples was that used previously (Kowalsky, 1962a). The expression pD = pH + 0.4 (Lumry *et al.*, 1951) was used to determine the acidity of the solutions. Extremely high concentrations of protein were necessary to obtain observable contact resonances, and concentrations were of the order of 0.015 M.

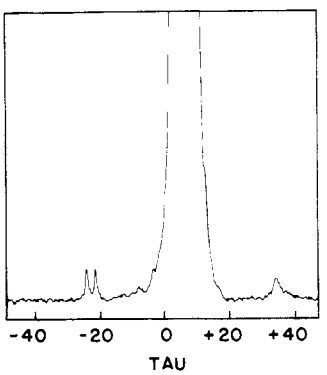

FIGURE 1: Nmr spectrum of ferricytochrome *c*: 0.02 M, pD 6.8.

Spectra were recorded at 60 Mc. It was not necessary to spin the samples. The contact spectra were generally obtained at fast scanning rates, although for the measurements of widths slower rates were used. However, saturation of the contact absorptions was not a problem at the power levels and sweep rates used.

Positions of the resonances are referred to the position of the methyl resonance of the sodium salt of 4,4-dimethyl-4-silapentane-1-sulfonic acid (Tiers and Kowalsky, 1960) and are expressed in terms of τ values (Tiers, 1958). Because of the compressed scale necessary to observe and record the contact spectra, it was impractical to actually use this reference in measuring positions. Therefore the main aliphatic resonance (CH_3CH_2) peak of cytochrome *c* was used. This relatively sharp resonance was given the value of τ 8.54 (from previous work), and positions were measured relative to this peak. In the heme peptide spectra, the main aliphatic peak used as a secondary reference was

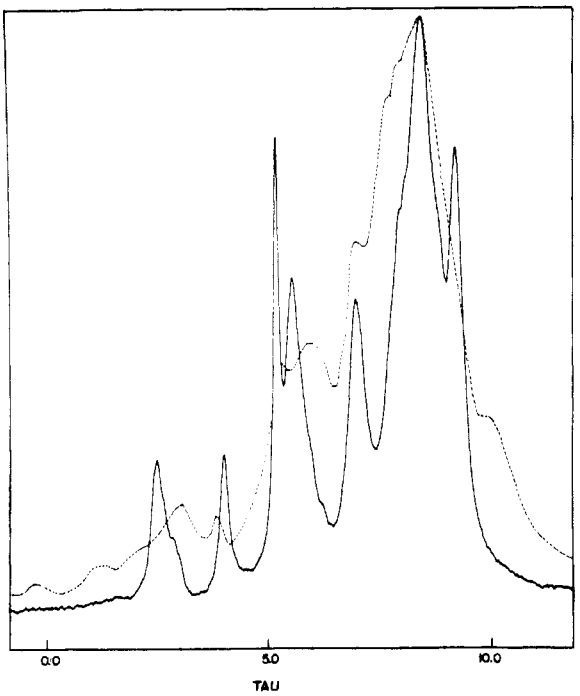

FIGURE 2: Nmr spectrum of ferricytochrome *c* in 8 M urea: 0.009 M, pD 7.0 (solid line). For comparison, the spectrum of ferricytochrome *c* in the absence of urea is drawn (dashed line). The sharp resonance in both spectra is that of HDO. The resonance at τ 4 in the urea spectrum arises from urea. The vertical scales are not the same.

found to be at τ 8.31. For the solutions of the hematohemin in pyridine-d_5, the reference used was tetramethylsilane (Tiers, 1958), and the τ scale was also used. Areas were measured with a planimeter.

Results

Cytochrome c. An examination of the proton magnetic resonance spectrum of oxidized cytochrome *c* at high radiofrequency power and amplification reveals the presence of three anomalous resonances well removed from the normal range of protein resonances (Figure 1). These resonances are seen only in the spectrum of the oxidized form of the protein and are completely absent in the spectrum of the reduced form, indicating that they may arise from the effect of the unpaired electron in the oxidized form. They are unaffected by the presence of ethylenediaminetetraacetate (EDTA), indicating that adventitious metal ions are not playing any part in the phenomenon. The positions of these resonances are listed in Table I.

An attempt was made to estimate the number of protons involved in each of the low-field absorptions. The aliphatic resonances of the protein and the heme group (from τ 5 to 10) of cytochrome *c* were calculated to represent 622 protons and were used as a standard.

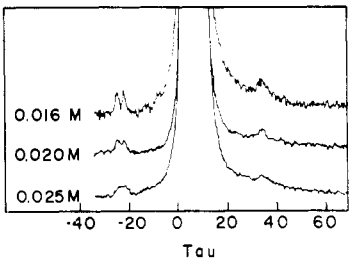

FIGURE 3: Variation of line width of contact resonances with ferro- and ferricytochrome *c* concentration: pD 7.0, 0.1 M phosphate, 0.1 M KCl. Concentrations given are total concentrations. All samples contained the same proportion of reduced form, about 50%.

The number of protons involved in each of the resonances at τ −21 and −24 was then calculated to be 3.7 protons. It was not possible to make similar calculations for the resonance at τ +33.9.

The positions of these resonances are invariant with pD from 5 to 9. At pD 10.4 there are three low-field resonances occurring at τ −13.3, −10.9, and −3.0. At higher pD values these are fused and nonresolvable. At these high pD values the spectrum of the aromatic protons is different from that of the native oxidized protein. The contact resonances are also absent in the spectrum of ferricytochrome *c* in acid media (at pD 1.4 or pD 3.1, 0.5 M KCl). At these low pD values nonexchangeable nitrogen-bonded protons are still present. Their resonances and those of the aromatic protons do not show the complex appearance characteristic of the spectrum of the native oxidized protein. In acid medium, the resonances in the τ 10 and 12 region (Kowalsky, 1962a) are also absent.

The presence of 8 M urea completely abolishes the contact spectra, indicating that the interaction responsible for these resonances is dependent on the configuration of the protein. The possibility that it is a change from polymer to monomer (Margoliash and Lustgarten, 1962) being observed is eliminated, since examination of the visible absorption spectrum of the ferricytochrome *c* showed that the band at 695 mμ characteristic of the monomer (Schejter *et al.*, 1963) was present with the correct intensity.

That some alteration in the structure of the protein has occurred in 8 M urea is shown by the main portion of the nuclear magnetic resonance (nmr)[1] spectrum of the protein (Figure 2) (Kowalsky, 1962b). It is of interest that the nmr spectrum of reduced cytochrome *c* in 8 M urea is unaltered from that of the native reduced protein (Kowalsky, 1962b). The change, in urea solutions, of the spectrum of the oxidized form raises again the possibility that there is a change in the electronic state of the iron rather than in the structure of the protein and that this is playing an important part in determining the shapes of the resonances. However, static mag-

[1] Abbreviation used: nmr = nuclear magnetic resonance.

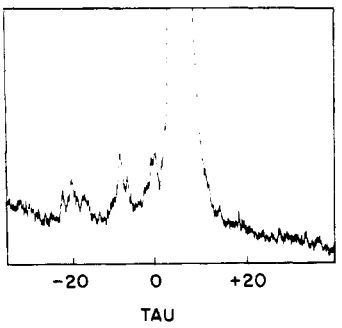

FIGURE 4: Nmr spectrum of the heme undecapeptide: 0.06 M, pD 10.4.

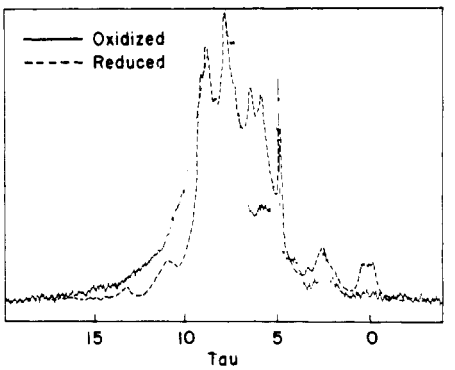

FIGURE 5: Nmr spectrum of the heme undecapeptide: 0.05 M + 0.05 M imidazole, pD 10.4.

netic susceptibility determinations using the nmr method (Reilly *et al.*, 1955; Li *et al.*, 1962) indicated that the iron in ferricytochrome *c* in 8 M urea is still in the low-spin state. The effect of urea is reversible; after the urea has been dialyzed out, all resonances previously seen in the spectrum of the native form, including the contact resonances, reappear, and the over-all shapes of the resonances are the same.

Contact resonances have also been observed in the same position in cytochrome *c* of pigeon breast muscle and of hog kidney although the main portions of these spectra differ from each other and from that of horse heart cytochrome *c*. They can also be seen in the spectrum of metmyoglobin. However, in the spectrum of the latter protein, there are four which occur over a much wider range, up to 80 ppm downfield of the main aliphatic proton resonance. In the spectrum of the paramagnetic-reduced form of myoglobin, these contact resonances shift to the immediate region of the main proton resonance.

The positions of the contact resonances in ferricytochrome *c* were found to be independent of the amount of reduced form present. From 0 to 23% reduced form was investigated. The widths of these resonances appeared to vary, however (Figure 3). Such studies were severely limited because the small absorptions required high concentrations of proteins, but at very high concentrations of protein there was a broadening due to a concentration effect. Thus it was possible to work only in a narrow range of concentrations.

Since the positions remain constant but the width varies, the rate of electron exchange between the two forms must be slow. An upper limit may be placed on this rate if it is assumed that the normal absorptions in the reduced form, unperturbed by any electronic factor, fall at τ 10. This rate is 10^5 M sec^{-1}. Measurements of the rate by varying concentration were not very precise because of the experimental difficulties mentioned; nevertheless a figure of $5 \pm 3 \times 10^4$ M sec^{-1} was obtained (pD 7.0, 0.1 M KCl, 0.1 M potassium phosphate). In these measurements, the broadening due to the concentration effect was corrected for.

Heme Peptides and Heme. In an effort to determine the location of the protons responsible for the contact shifts, the heme undecapeptide, the heme octapeptide, and the hematohemin itself, all derived from cytochrome *c*, were studied. The peptides are still cyclically linked to the porphyrin ring and contain groups capable of coordinating with iron. Contact shifts were found in all these spectra, but they were not of the same shape nor were they found at the same positions. The positions of these resonances (Table I) were sensitive to experimental conditions and could not be determined with high accuracy.

Figure 4 shows the spectrum of the undecapeptide at pD 10.4. The resonances do not appear in the spectrum of the reduced form of the peptide, again indicating that they arise from an interaction of certain protons with the unpaired electrons. The positions and the shapes of these resonances are pH dependent. Sharp resolved resonances are observed at pD values 10.9 and 11.9. As the pD is lowered, the absorptions become progressively broader and shift toward higher fields. At pD 7.9, they become a broad band fused to the main peak. The transition from one extreme to the other does not coincide exactly with the interconversion of a nonprotonated lysine chelated to the iron and a protonated but nonchelated lysine, pK = 10.5 (Harbury and Loach, 1960). It seems to occur over a broader range at somewhat lower pH values; by the time the pD corresponding to the pK of the lysine transition is reached (10.5), the spectral change seems almost complete.

The peptide was also observed in the presence of imidazole. The undecapeptide associates in solution (Harbury and Loach, 1959). Histidine is known to cause dissociation. In approximately equimolar amounts, however, contrary to expectation, imidazole did not result in increased resolution. Rather, the absorptions became broader and resolution decreased. Above pD 10.5, the imidazole would also coordinate with the iron. There would be at least three species of peptide present in solution, and exchange among these various forms of peptide might possibly cause the broadening. In all these cases the imidazole resonance could be seen at about τ 2.5.

The main portions of the spectra of the heme undeca-

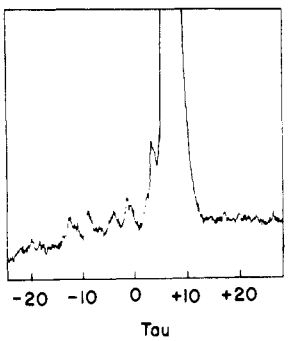

FIGURE 6: Nmr spectrum of the heme octapeptide: 0.03 M, pD 9.5. This sample contained a small amount of pyridine-d_5.

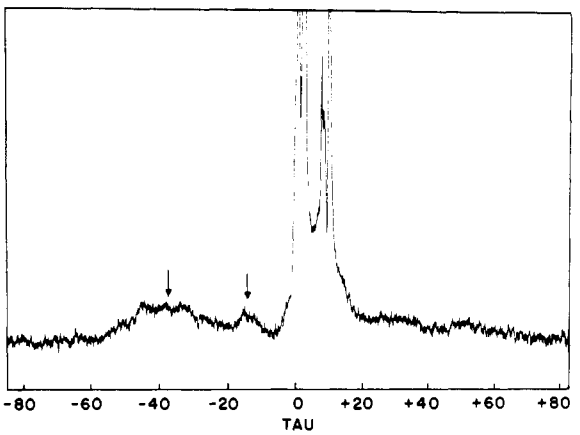

FIGURE 7: Nmr spectrum of hematohemin of cytochrome *c*: 5% solution.

peptide, oxidized and reduced, in the presence of imidazole are presented in Figure 5. In the spectrum of the reduced heme peptide, the resonances at about τ 6 arise from the protons α to carboxyl and amino groups in the peptide (Jardetzky and Jardetzky, 1958), and from the methyl and methylene proton substituents on the porphyrin ring (Becker *et al.*, 1961; Abraham *et al.*, 1961; Caughey and Koski, 1962); the resonances at τ 2.5 arise from the histidine and added imidazole, and those at about τ 0 from porphyrin ring methine bridge protons. Note that, in the oxidized form, the absorptions at τ 0 are completely absent and those at τ 6 are decreased considerably. It is thus probable that the methine bridge protons of the porphyrin ring are the origin, at least in part, of the peptide contact resonances. In addition, α peptide protons or porphyrin ring methyl and methylene protons may also, in part, be the source of the contact peaks in the undecapeptide spectra. In 8 M urea, the spectrum of the undecapeptide showed contact peaks in the same approximate positions but with altered shapes (Table I).

Similar contact resonances were observed in the spectrum of the heme octapeptide (Figure 6). However, they did not fall in the same positions as those of the undecapeptide (Table I). Since this octapeptide does not contain lysine, the presence of the contact resonances in the octapeptide spectrum would indicate that lysine protons are not responsible for these absorptions. The pH behavior of the resonances appeared to be different from those of the undecapeptide but was not investigated in detail.

The hematohemin of cytochrome *c* was examined in pyridine-d_5 (Figure 7). Here the contact resonances no longer consist of discrete absorptions but appear as two very broad complex resonances. In a solvent consisting of 0.2 N KOD, 20% pyridine-d_5, no anomalously shifted resonances could be seen.

Discussion

The explanation for the appearance of the contact resonances in the spectrum ferricytochrome *c* is not entirely clear, but certain sources may be excluded. The positions of the lines preclude the resonances being a normal absorption of an amino acid proton which occurs over the range τ 0–10 (Jardetzky and Jardetzky, 1958). The large porphyrin ring current does shift the resonances of substituent protons to abnormally high and low fields (Becker *et al.*, 1961), but the contact shifts are beyond this range. Furthermore the ring current effect would be operative in the reduced form of the protein also, and the spectrum here shows no contact shift.

Cytochrome *c* does contain about 2% of the high-spin form (George *et al.*, 1963). However, since the resonances are not seen when the protein is in acid solution, and in this medium the iron is completely in the high-spin state, this form of the protein is not responsible for the resonances. These resonances then must arise from an interaction of certain protons with the one unpaired electron of the iron. The two possible mechanisms by which this interaction can take place are: isotropic hyperfine contact interactions (Fermi, 1930), or pseudo-contact interactions (Bloembergen and Dickinson, 1950; McConnell and Robertson, 1958). It is difficult to differentiate between the two types of interaction, but some deductions can be made concerning these mechanisms from the direction of the shift.

Isotropic Hyperfine Contact Interaction. In a spin-paired system such as is present in ferricytochrome *c*, the five d electrons of the central ion will distribute themselves among the three lower t_{2g} orbitals, and one electron will have its spin unpaired. The t_{2g} orbitals have π-type symmetry, and electrons occupying them are not available for σ bonding to the ligand. Delocalization of the magnetic electron in a π system through a molecular orbital is possible; *i.e.*, the unpaired electron may be found to some extent on the nitrogen of the ligand. Once on the nitrogen, the unpaired spin can be propagated through the ligand carbon skeleton either through (a) σ bonds of the ligand (through π–σ interaction) or (b) through the π molecular orbitals in which the electron is already present.

Transmission of the spin through the σ-bond system

places nonzero spin densities on carbon atoms of the ligand, and these can be transmitted also through the σ system to adjacent bonded hydrogen nuclei. The effect of the unpaired spin acting directly at the nucleus is to shift the resonance far from the normal position. This mechanism would require positive spin densities at ligand protons, thus shifting all proton resonances to lower fields. However, in the spectrum of cytochrome *c*, shifts to both high and low fields are observed. Thus, transmission of the unpaired spin *via* σ bonds in the prosthetic group or in the protein is eliminated as the sole mechanism. Such considerations do not hold for the heme peptides or the hematohemin derived from cytochrome *c*, for all shifts in these compounds are to low field. It is possible that in these derivatives the shifts may occur solely by σ-bond transmission of the unpaired spin.

If the spin is transmitted by the π-electron system, then some π–σ interaction is still necessary at the carbon atom for the unpaired spin to reach the proton. The expression for the magnitude of the hyperfine contact interaction (McConnell and Chesnut, 1958) indicates that shifts can be either to high or low field according to whether the spin density is negative or positive. Such contact interactions have been observed in systems of nickel chelates (Eaton *et al.*, 1962).

Pseudo-Contact Interaction. Alternatively, shifts of proton resonances may occur through a pseudo-contact interaction. Here, a dipolar type of interaction between metal ion and proton is not averaged out to zero by molecular tumbling because of the anisotropy in the *g* value of the complex. The shift is given by

$$\frac{\Delta H_i}{H} = \frac{-\beta^2 S(S+1)(3\cos^2\phi_i - 1)(g_{\parallel} + 2g_{\perp})(g_{\parallel} - g_{\perp})}{27 r_i^3 kT} \quad (1)$$

where r is the distance between the unpaired electron and the ith nucleus, ϕ is the angle between the crystal field axis of the complex and the radius vector from the metal ion to the nucleus, and $g_{\parallel}$ and $g_{\perp}$ are the electronic g factors parallel and perpendicular to the crystal field axis. Pseudo-contact shifts have been observed in Co(II) chelates (Happe and Ward, 1963).

The electron spin resonance of cytochrome *c* has been investigated (Gordy and Rexroad, 1961), and two *g* values were found, indicating the anisotropy of this factor. With the aid of eq 1, a pseudo-contact shift may be calculated for the methine bridge protons of the porphyrin ring to be of the order of 2 ppm to high field. Thus it does not seem likely that the anomalous resonances of the ferricytochrome *c* spectrum arise from a pseudo-contact interaction with these porphyrin protons. However, protons of the protein itself, held or forced much closer to the iron, could conceivably be involved in this type of interaction.

It should be noted that ^{14}N resonance shifts have been observed (Shulman, 1958) in the spectrum of the low-spin ferricyanide ion; here the iron is formally analogous to the iron in ferricytochrome. In this case, it was assumed that the shifts were the result of pseudo-contact interactions.

Considering the fact that the iron is surrounded not only by the heme with its various types of protons but also by the protein containing its own numerous protons, one would expect to see, if this were a pseudo-contact interaction, not just a simple pattern of two low-field resonances and one high-field resonance, but a complete complex series of resonances. The simplicity of the contact spectra argue for a true hyperfine contact interaction.

Electron Exchange. The rate of electron exchange between oxidized and reduced cytochrome *c* is extremely low when compared with the rate of oxidation of the protein by ferricyanide (10^7 M sec^{-1}) (Sutin and Christman, 1961). However, a low rate of electron exchange in such a system is not without experimental precedent. The rate of electron exchange in the ferrous–ferric system and in the ceric–cerous system is very slow. Yet, the rate of ceric oxidation of ferrous ion is very fast. Furthermore, the rate of ferrocyanide–ferricyanide electron exchange is itself low, about 350 M sec^{-1}.

Heme Peptides and Hematohemin. In comparing the heme peptide and heme derivatives of ferricytochrome *c*, it is important that analogous states of iron bonding be considered (see Table I). The static magnetic susceptibility of the 11-membered heme peptide has been measured (Paléus *et al.*, 1955). In alkaline solution, it is spin paired with one unpaired electron similar to cytochrome *c*. The observations reported here were carried out on alkaline solutions or alkaline solutions close to neutrality.

Since the shifts of the resonances of the heme peptides are all to low field, the previous calculation of a positive pseudo-contact shift for the methine bridge protons makes it doubtful that this type of interaction is operative. However, there is a serious question as to whether the shifts observed in ferricytochrome *c* spectra, whatever their origin, are the same as those observed in the heme peptides. Results obtained for ferricytochrome *c* in the presence of 8 M urea indicate that the contact resonances are dependent on protein conformation. Yet the addition of 8 M urea to the heme undecapeptide causes no marked change in the anomalous resonances. There may not be the same tertiary structural requirement in the heme undecapeptide for interaction resulting in the observed shifts.

In the case of the heme derivative, the question of type of metal bonding also occurs. When the cytochrome *c* hematohemin was examined in 20% pyridine–0.2 N NaOD solvent, no contact resonances could be found. Such absorptions, however, could be seen when pure pyridine was the solvent. It is surprising that no contact shifts occur when the compound is in the low spin state as is the case with the peptides. The hematohemin, similar to hemin, may be aggregated in solution. This may result in pseudo-contact interactions between molecules in an aggregate.

Recently, shifts to both high and low fields have been

observed in a related compound, ferric tetraphenylporphyrin chloride, in $CDCl_3$ solution (Eaton and LaLancette, 1964). This compound was found to be spin free, having 5 unpaired electrons. These shifts were interpreted in terms of electron delocalization and hyperfine contact interactions.

At the present time, it is not possible to decide which of the two types of interaction in the protein and its derivatives is responsible for the anomalous resonances. The directions of the shifts cannot unequivocally eliminate either mechanism. Thus, the low-field shifts in the spectra of the peptide and hematohemin are consistent with a hyperfine contact interaction with σ-bond transmission of the unpaired spin. They can also be explained by a pseudo-contact interaction, although here calculations show they cannot arise from such an interaction with the methine bridge protons of the porphyrin ring. For the protein itself, the shifts to both high and low field also allow either possibility. The fact that only three absorptions are seen indicates a true hyperfine contact interaction.

Acknowledgment

The author wishes to acknowledge many helpful discussions with Dr. Mildred Cohn and the able assistance of Mr. J. S. Leigh in maintaining the equipment.

References

Abraham, R. J., Jackson, A. H., and Kenner, G. W. (1961), *J. Chem. Soc.*, 3468.

Becker, E. D., Bradley, R. B., and Watson, C. J. (1961), *J. Am. Chem. Soc. 83*, 3743.

Bloembergen, N., and Dickinson, W. C. (1950), *Phys. Rev. 79*, 179.

Bovey, F. A., Tiers, G. V. D., and Filipovich, G. (1959), *J. Polymer Sci. 38*, 73.

Caughey, W. S., and Koski, W. S. (1962), *Biochemistry 1*, 923.

Eaton, D. R., Josey, A. D., Phillips, W. D., and Benson, R. E. (1962), *Discussions Faraday Soc. 34*, 77.

Eaton, D. R., and LaLancette, E. A. (1964), *J. Chem. Phys. 41*, 3534.

Fermi, E. (1930), *Z. Physik 60*, 320.

George, P., Mullins, J., and Schejter, A. (1963), Abstracts, 7th Meeting of the Biophysical Society, p. TA 9.

Gordy, W., and Rexroad, H. N. (1961), *in* "Free Radicals in Biological Systems," Blois, M. S., Jr., Lindblom, R. O., Brown, H. W., Lemmon, R. M., and Weissbluth, M., eds., New York, Academic, p. 263.

Hagihara, B., Morikawa, I., Tagawa, K., and Okunuki, K. (1958), *Biochem. Prepn. 6*, 1.

Happe, J. A., and Ward, R. L. (1963), *J. Chem. Phys. 39*, 1211.

Harbury, H. A., and Loach, P. A. (1959), *Proc. Natl. Acad. Sci. 45*, 1344.

Harbury, H. A., and Loach, P. A. (1960), *J. Biol. Chem. 235*, 3640.

Jardetzky, O., and Jardetzky, C. D. (1957), *J. Am. Chem. Soc. 79*, 5322.

Jardetzky, O., and Jardetzky, C. D. (1958), *J. Biol. Chem. 233*, 383.

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

Kowalsky, A. (1962b), Abstracts, 142nd National Meeting of the American Chemical Society, Atlantic City, N. J., Sept. 1962, p. 10R.

Kowalsky, A. (1964), *Federation Proc. 23*, 222.

Li, N. C., Scruggs, R. L., and Becker, E. D. (1962), *J. Am. Chem. Soc. 84*, 4650.

Lumry, R., Smith, E. L., and Glantz, R. R. (1951), *J. Am. Chem. Soc. 73*, 4330.

Margoliash, E., and Lustgarten, J. (1962), *J. Biol. Chem. 237*, 3397.

McConnell, H. M., and Chesnut, D. B. (1958), *J. Chem. Phys. 28*, 107.

McConnell, H. M., and Robertson, R. E. (1958), *J. Chem. Phys. 29*, 1361.

Paléus, S., Ehrenberg, A., and Tuppy, H. (1955), *Acta Chem. Scand. 9*, 365.

Paul, K. G. (1950), *Acta Chem. Scand. 4*, 239.

Reilly, C. A., McConnell, H. M., and Meisenheimer, R. C. (1955), *Phys. Rev. 98*, 264A.

Schejter, A., Glauser, S. C., George, P., and Margoliash, E. (1963), *Biochim. Biophys. Acta 73*, 641.

Shulman, R. G. (1958), *J. Chem. Phys. 29*, 945.

Sutin, N., and Christman, D. R. (1961), *J. Am. Chem. Soc. 83*, 1773.

Tiers, G. V. D. (1958), *J. Phys. Chem. 62*, 1151.

Tiers, G. V. D., and Kowalsky, A. (1960), Abstracts, 137th National Meeting of the American Chemical Society, Cleveland, Ohio, April 1960, p. 17R.

Tuppy, H., and Paléus, S. (1955), *Acta Chem. Scand. 9*, 353.