was dissolved in benzene–Skellysolve B (1:1), adsorbed onto a column of Woelm neutral alumina (6 g, undeactivated), and left on the column for 11 hr. Elution of the column with chloroform (300 ml) gave crude monoketone XVI (59 mg). The crude material was then chromatographed on Woelm neutral alumina (15 g, deactivated with water, 4% v/w). Elution with Skellysolve B–benzene (4:1, 300 ml) gave a mixture (3 mg) with spectral properties corresponding to diethylene ketal derivatives. Further elution with Skellysolve B–benzene (3:2, 100 ml) gave the desired monoketone XVI (20 mg). Crystallization of this material from Skellysolve C gave pure XVI: mp 118–120°; $[\alpha]^{26}D$ +72° (c 0.032, methanol); λ_{max} 5.86 μ; nmr, τ 9.38 (3 H, s), 7.90 (3 H, s), 6.05 (4 H, s).

Anal. Calcd for $C_{23}H_{36}O_3$: C, 76.62; H, 10.06. Found: C, 76.38; H, 10.46.

The next volume (120 ml) of Skellysolve B–benzene (3:2) yielded three-spot material (11 mg). Finally, elution with Skellysolve B–benzene (3:2, 125 ml) and with benzene (100 ml) gave some diketone (22 mg).

9(10→19)*abeo*-5α,10α-Pregnane-3,20-dione (XVIII). **a.** 9(10→19)*abeo*-$\Delta^{9(11)}$-5α,10α-Pregnene-3,10-dione 3,20-diethylene ketal (X, 70 mg) was hydrogenated as described above to yield crude product (68 mg). A solution of this product and *p*-toluenesulfonic acid monohydrate (5 mg) in water (1 ml) and acetone (4 ml) was refluxed for 3.5 hr and then left at room temperature overnight. The acetone was removed under reduced pressure at room temperature and the remaining residue was treated with water (5 ml) and benzene (10 ml). The organic layer was separated and the aqueous layer was extracted twice with chloroform–benzene (1:2, 10 ml). The combined organic layer was washed with saturated salt solution and dried over anhydrous magnesium sulfate. After filtration the solution was evaporated to dryness to leave a colorless oil (50 mg). This oil was combined with the diketone (22 mg) obtained during preparation of the monoketone. The combined material was chromatographed on Woelm neutral alumina (15 g, deactivated with water, 3.5% v/w). Elution with Skellysolve B–benzene (1:1, 220 ml) gave a mixture (30 mg) containing some of the desired diketone. Further elution with Skellysolve B–benzene (1:2, 90 ml) furnished crystalline XVIII (6 mg). Recrystallization from *n*-hexane provided pure XVIII (4 mg): mp 149–150°; $[\alpha]^{26}D$ +94° (c 0.015, methanol); λ_{max} 5.88 μ; nmr, τ 9.38 (3 H, s), 7.90 (3 H, s).

Anal. Calcd for $C_{21}H_{32}O_2$: C, 79.70; H, 10.19. Found: C, 79.51; H, 10.01.

b. A solution of 9(10→19)*abeo*-5α,10α-pregnane-3,20-dione 3-monoethylene ketal (XVI, 10 mg) and *p*-toluenesulfonic acid monohydrate (2 mg) in acetone (3 ml) and water (0.3 ml) was refluxed for 3 hr. Acetone was removed by evaporation under reduced pressure at room temperature and the residue was treated with water (3 ml) and extracted with three 5-ml portions of benzene. The benzene extract was washed once with water and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to leave a gray residue (6 mg). This residue on crystallization from Skellysolve C provided diketone XVIII (2 mg), mp 147–150°. The melting point was not depressed upon admixture with the diketone prepared by method a, and the thin layer chromatographic behavior of the materials were identical.

Manifestations of the Tertiary Structures of Proteins in High-Frequency Nuclear Magnetic Resonance

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Contribution No. 1284 from the Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898.
Received June 19, 1967

Abstract: Manifestations in high-frequency (220 Mcps) proton magnetic resonance spectroscopy of the conformational differences that exist between the native and denatured forms of ribonuclease, lysozyme, and cytochrome *c* are described. High-field resonances in the 0.7- to −1.0-ppm range (relative to the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate) are exhibited by lysozyme and cytochrome *c only* in the folded, native conformations. The unusual positions of these resonances are attributed to high-field ring current shifts that are induced in the highly shielded C–H protons of such residues as leucine, isoleucine, valine, lysine, methionine, arginine, threonine, and alanine whose side chains are proximal to the faces of the aromatic groups of the histidine, phenylalanine, tyrosine, and tryptophan residues in the proteins' folded conformations. Additional, even larger diamagnetic shifts (in the −2.0- to −3.8-ppm range) are observed in the pmr spectra of the oxidized and reduced forms of folded cytochrome *c* that are believed to derive from side chains of component amino acids that experience the large ring current field associated with the extensively conjugated porphyrin ring. Pmr spectral differences between ferricytochrome *c* and ferrocytochrome *c* strongly suggest that important conformational dissimilarities exist between the oxidized and reduced forms of the protein that are due to differences in side-chain coordination at the heme iron. High-field resonances characteristic of the folded conformations are also observed for native myoglobin and hemoglobin. One of these that occurs at −3.7 ppm in the spectrum of myoglobin has a resonance width of 110 cps. This width is attributed to paramagnetic relaxation by the high-spin heme iron. Conversion of myoglobin to the low-spin form as the result of coordination of CN^- to the heme iron leads to the appearance of two sharp resonances in the −3.0- to −3.3-ppm region of the spectrum.

There have been a number of studies of proteins in solution by proton magnetic resonance (pmr) spectroscopy.[1-11] Structural information derivable from such studies has been severely limited by the overlap of chemical shifts of the very large number of structurally

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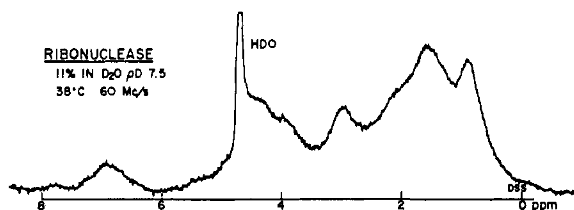

Figure 1. 60-Mcps pmr spectrum of ribonuclease.

and environmentally nonequivalent protons encountered in these molecules in their native, folded conformations in neutral aqueous solution. Since chemical shifts are linear functions of the polarizing magnetic field employed, it is of obvious advantage to investigate proteins at the highest field (and concomitant frequency) possible. Most previous pmr studies on proteins have been carried out at resonance frequencies of 100 Mcps or less. We wish to report here some pmr characteristics of proteins observed using a 220-Mcps spectrometer. A preliminary report of some of the results to be discussed below has appeared.[12]

Experimental Section

Proton magnetic resonance (pmr) spectra were obtained on a Varian Associates high-resolution spectrometer which operates at a frequency of 220 Mcps. The polarizing field of 52,000 gauss is furnished by a superconducting solenoid.[13,14] The temperature in the sample zone is established by a stream of nitrogen of controlled but variable temperature and was regulated within $\pm 1°$ in our experiments. Internal referencing with sodium 2,2-dimethyl-2-silapentane-5-sulfonate was employed throughout. This resonance appears at 0 ppm in several of the spectra presented here. Chemical shifts are expressed in units of parts per million (ppm) from this reference resonance, with the convention that shifts to low field are assigned positive values. Signal-to-noise ratios were improved in some instances by employing a computer of average transients (Varian Associates C 1024). The proteins employed in these studies were the best commercially available. The trimer of N-acetylglucosamine employed in the lysozyme inhibition studies was provided by Dr. J. A. Rupley.

Results and Discussion

Ribonuclease. The 60-Mcps pmr spectrum of ribonuclease dissolved in D_2O is shown in Figure 1. The spectrum is similar to that obtained by Saunders and co-workers[1] in their pioneering study at 40 Mcps. The resolution that has been achieved is not impressive and at these frequencies differences in the pmr spectra of ribonuclease in native and denatured conformations are difficult to discern, and certainly impossible to describe in any quantitative sense. Pmr absorption of ribonuclease at 220 Mcps in the native and thermally denatured conformations are shown, respectively, in the top and bottom spectra of Figure 2. The strong line in the 4.5–5.5-ppm range in these and subsequent spectra arises from HDO. Weaker, sharp resonances on either side of this resonance in some of these spectra are spinning side bands. The 220-Mcps pmr spectrum of ribonuclease is fairly well resolved, at least in comparison with the 60-Mcps spectrum, even in the folded conformation. A most striking simplification and improved resolution is achieved, however, upon thermal denaturation. It thus would appear that 220-Mcps pmr spectroscopy possesses a useful potential for the study of protein conformation in solution.

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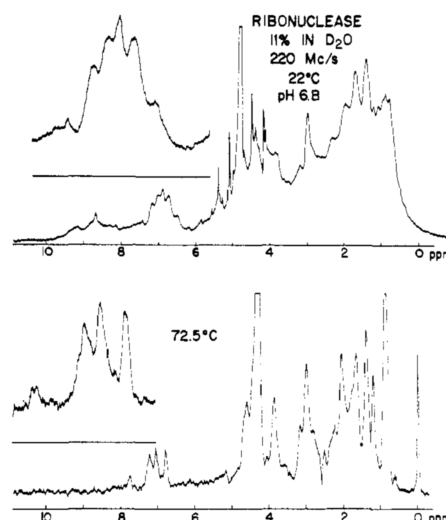

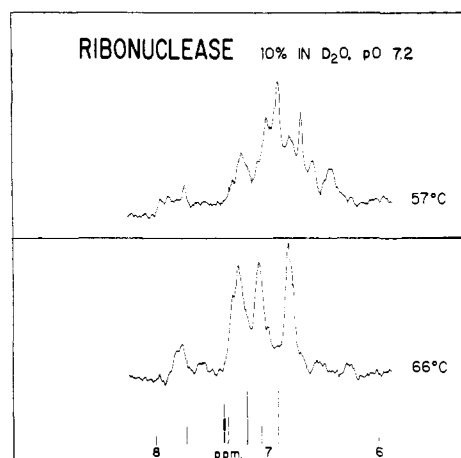

Figure 2. 220-Mcps pmr spectra of ribonuclease in native (top) and denatured (bottom) conformations.

Figure 3. Radiofrequency absorption at 220 Mcps in the "aromatic" region of the pmr spectrum of ribonuclease (57°, native; 66°, denatured).

Fair progress in the analysis of the pmr spectrum of ribonuclease in the denatured form can be made on the basis of the expected positions of resonances of the component amino acids.[4-12] For example, the sharp, extreme high-field resonance that occurs at 0.85 ppm in the 72.5° spectrum of Figure 2 can be attributed to the highly shielded methyl protons of valine, leucine, and isoleucine. Of the aromatic amino acids, ribonuclease contains four histidine, three phenylalanine, and six tyrosine residues; tryptophan is absent. Resonances appearing in the 6.8–7.8-ppm region of the spectrum of the denatured form of the protein can be assigned to the aromatic protons of these component amino acids. Positions and relative intensities of resonances for the mixture of aromatic amino acids contained by ribonuclease are indicated by the lines beneath the 66° spectrum of Figure 3. On this basis, the 7.8-ppm resonance is attributed to the C-2 protons of histidine; the phenyl protons of phenylalanine appear as the broadened peak at 7.3 ppm; the *ortho* and *meta* protons of the phenyl group of the tyrosine residues are associated, respectively, with the 6.8- and 7.2-ppm

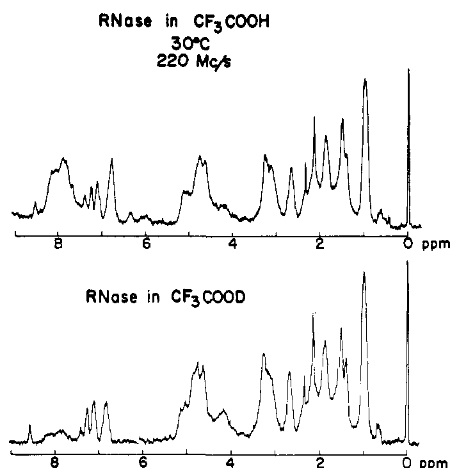

Figure 4. Pmr spectra of ribonuclease in CF_3COOH and CF_3-COOD.

resonances. With the exception of the C-2 protons of histidine, resonances of aromatic protons are shifted about 0.15 ppm to high field upon incorporation of the amino acid into the polypeptide chain.

Pmr spectra of ribonuclease in the denaturing solvents CF_3COOH and CF_3COOD are shown in Figure 4. A general observation to be made with regard to these spectra is that, while there is by no means a one-to-one correspondence between resonance positions and intensities for ribonuclease dissolved in trifluoroacetic acid and the protein in D_2O solution above the denaturation temperature, line widths and spectral simplicity are comparable in the two solvents. Differences between the spectra of ribonuclease in D_2O above 65° and in trifluoroacetic acid can be attributed largely to chemical shifts that accompany protonation of the enzyme in the acid medium. Some additional low-field resonances are observed when CF_3COOH is used as solvent because the rates of exchange of N–H and O–H protons have been reduced to the level that separate, sharp resonances are observed for these protons. In CF_3-COOD, such protons are replaced by deuterons and their contributions to the spectrum of ribonuclease in trifluoroacetic acid are no longer present.

It appears then that in the denatured state the side chains of component amino acids of proteins are largely unconstrained and consequently exhibit chemical shifts for the various structurally nonequivalent protons that do not strongly reflect the amino acid sequence of the protein. Nearest neighbor interactions and conformational proximity effects do not appear to play a major role in determining the chemical shifts of the side-chain protons. Consequently, all residues of a given amino acid give rise to a spectrum of resonances very similar to that of the free amino acid so that the pmr absorption of the denatured form of the protein can be rather well represented by a superposition of the resonances of the component amino acids, weighted by the amino acid composition of the protein.

The pmr spectra of ribonuclease in D_2O at temperatures below 65° (the denaturation temperature) are, however, markedly different from those of the enzyme in CF_3COOH or in D_2O above the denaturation temperature. Many new resonances appear and the intensity distribution of resonances over the spectrum is grossly

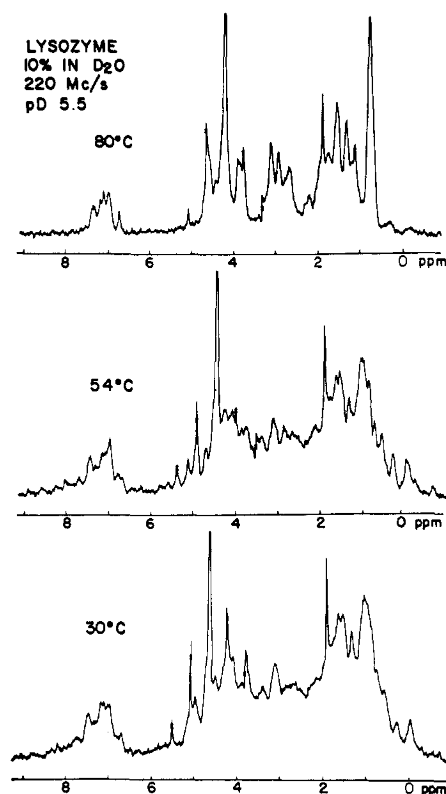

Figure 5. Temperature dependence of the 220-Mcps pmr spectrum of lysozyme. The HDO resonance is in the 4.3–4.6-ppm range. Spinning side bands are indicated by X.

altered. In the native form of the protein, side-chain mobility seems to be greatly constrained and, on the average, side chains are much closer together or at least more environmentally perturbed, in the nmr sense, than in the random coil form. Consequently, in the folded conformation, electrical and magnetic effects of short-range nature can produce quite appreciable chemical shifts. In the folded conformation the resonances of a single amino acid of a protein reflect its local environment. Thus, the spectrum of the native protein is very much more complex, and at the same time potentially much more revealing than that of its random coil form.

Thus, as RNase and, as will be seen, other proteins are cooled a few degrees through their classical thermal renaturation ranges, new pmr spectra emerge, profoundly different from those of the random coil forms, that are characteristic of the folded proteins. Minor continuous changes in these latter spectra occur over a wide temperature range as the protein solutions are further cooled, indicating the occurrence of further small, but real, conformational readjustments. Thus, by carefully following pmr changes as a function of temperature, it should be possible to monitor protein conformational changes associated with denaturation or renaturation. In a similar vein, it would be hoped that the approach will possess sufficient sensitivity to reflect whatever conformational changes that may accompany the interactions of proteins with substrates, effectors, inhibitors, nucleic acids, lipids, and metal ions. In the present discussion, however, we will be concerned primarily with manifestations in nmr of temperature effects on the conformations of proteins.

Lysozyme. The temperature dependence of the pmr spectrum of lysozyme is shown in Figure 5.

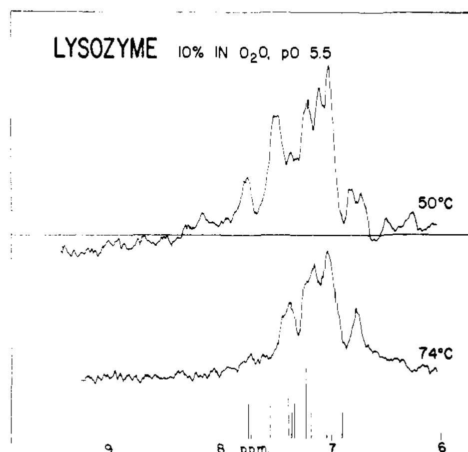

Figure 6. Radiofrequency absorption at 220 Mcps in the "aromatic" region of the pmr spectrum of lysozyme (50°, native; 74°, denatured).

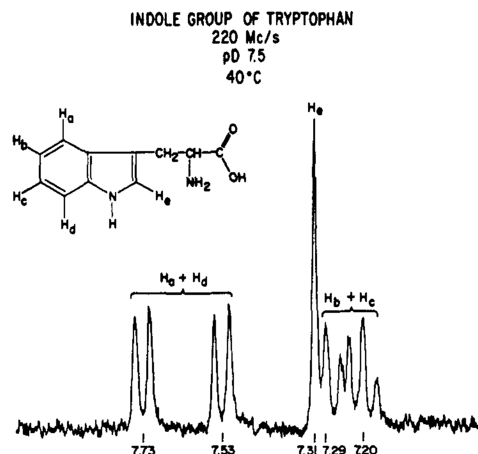

Figure 7. Pmr absorption of the indole group of tryptophan.

Above 74° where, under the environmental conditions employed in this study, thermal denaturation is complete, the pmr spectrum of lysozyme exhibits the relatively simple, sharp-line spectrum that was found characteristic of denatured ribonuclease. Expansions of the low-field region of absorption of the aromatic protons of lysozyme in the native (50°) and denatured (74°) conformations are presented in Figure 6. Detailed analysis of this region of the spectrum even in the "simplified" spectrum of the denatured form is complicated by the relatively high tryptophan content of the protein. Out of a total of 129 amino acids, egg-white lysozyme contains six tryptophan residues.[15,16] The complex nature of the absorption of the indole protons of tryptophan in the aromatic region (Figure 7) is in striking contrast to the simplicity of absorption of the aromatic protons of histidine, phenylalanine, and tyrosine. In any event, the spectrum of lysozyme in this region based on its aromatic amino acid content is indicated below the 74° spectrum of Figure 6, and when shifted 0.2–0.3 ppm to high field, is seen to be in fair qualitative agreement with the observed spectrum of the denatured form of the protein.

The most interesting manifestations in pmr of the conformational changes that accompany denaturation of lysozyme are observed in the extreme high-field region of the spectrum. In the denatured form of the protein (the 80° spectrum of Figure 5) the sharp, intense high-field resonance that occurs at 0.85 ppm is assigned, as in the case of ribonuclease, to the highly shielded methyl groups of leucine, isoleucine, and valine. As, however, has been reported earlier[12] and as is seen in Figures 5 and 8, the reversible reassumption of the native conformation of the protein that accompanies a reduction of temperature to below 74° results in a decrease in intensity of this resonance with a concomitant emergence of resonances in the 0.7- to −0.7-ppm range. Appearance of resonances in this region is noteworthy since none are found here in the spectra of component amino acids nor in the spectra of lysozyme or other proteins so far examined *in the denatured form.* These resonances then would appear to reflect in rather sensitive fashion aspects of the folded conformation of the protein. Consequently, it is of some interest to inquire into the origin of these high-field resonances in the expectation that they will be of value in elucidating, for example, interactions of the enzyme with substrate and inhibitor and effects of environment on the conformation of the enzyme.

It is well established that aromatic rings are capable of inducing large, through-space chemical shift effects known as ring current shifts.[17] The phenomenon owes its existence to the fact that under the influence of an external magnetic field the mobile π electrons of an aromatic ring give rise to a small magnetic field that can either add to or subtract from the external field in varying amounts, the magnitude of the ring current field depending on the orientation and distance of the affected nucleus with respect to the aromatic ring.[18–20] Particularly striking examples of ring current shifts have been observed in the pmr spectra of porphyrins.[21,22] Ring current fields shift the resonances of protons situated on the periphery but exterior to the plane of aromatic groups to low field because in this configuration the ring current field adds to the external polarizing field. On the other hand, protons located above and below the planes of aromatic rings experience high-field shifts since in this orientation the ring current field opposes the external field.

Johnson and Bovey[20] have carried out calculations based on a ring current model of the magnetic field associated with the six π electrons of benzene. To illustrate the magnitudes of the fields and ring current shifts involved, a few selected shifts to be expected at points above and below the plane of a benzene ring are presented in Table I. The diamagnetic ring current fields are fairly constant to radial distances of 1 A, but as might be expected decrease rapidly along the sixfold axis. The minimum approach of the protons of, for example, a methyl group to a benzene ring along the sixfold axis of the ring would be about 3.0 A and in this approach the protons should be subjected to a diamagnetic ring current field equivalent to a shift of about 1.8

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(16) See, for example, B. Jirgensons, "Natural Organic Macromolecules," Pergamon Press Inc., New York, N. Y., 1962.
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Table I. Calculated Ring Current Shifts[a] Associated with Benzene[b]

ρ, A[d]	z, A[c] 0	1.11	2.22	2.78	3.34	4.17	4.86	5.56
0	−15.22	−11.03	−3.75	−2.18	−1.34	−0.72	−0.46	−0.31
0.28	−15.26	−11.08	−3.66	−2.13	−1.32	−0.71	−0.45	−0.31
0.56	−15.23	−11.20	−3.41	−2.00	−1.25	−0.68	−0.44	−0.30
1.11	−11.72	−9.66	−2.47	−1.55	−1.02	−0.59	−0.40	−0.28
2.22	+1.73	+1.01	−0.51	−0.54	−0.47	−0.34	−0.26	−0.20

[a] Ring current shifts expressed in parts per million. The negative sign denotes a diamagnetic field and the positive sign a paramagnetic field, high and low resonance field shifts, respectively. [b] See ref 20. [c] Distance along the sixfold axis. [d] Radial distance in the plane of the ring.

ppm; indeed, ring current shifts of at least 1.4 ppm are experienced by certain methyl or methylene groups of lysozyme in the protein's folded conformation. As will be seen later, ring current fields of up to 4.5 ppm are experienced by side-chain protons of cytochrome *c* and these must be attributed to the larger fields associated with the more extensively delocalized porphyrin ring.[21,21]

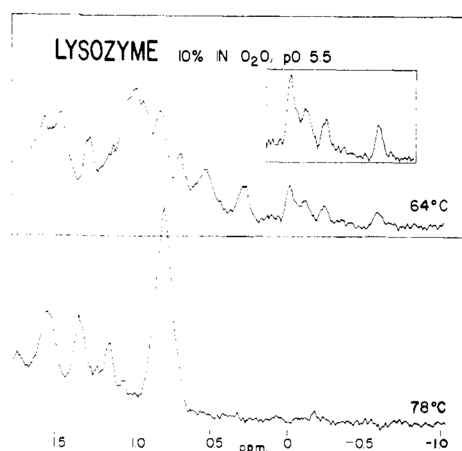

Figure 8. Radiofrequency absorption at 220 Mcps in the high-field region of the pmr spectrum of lysozyme (64°, native; 78°, denatured).

It is most tempting to consider the possibility of applying calculated ring current shifts to the analysis of the high-field regions of the spectra of proteins such as lysozyme and cytochrome *c* and in so doing specify a number of side-chain contact points for their folded conformations. However, agreement between theoretical and experimental *in-plane* ring current shifts for porphyrins and molecules containing phenyl rings have been at best semiquantitative.[23] For lack of suitable test molecules, calculated ring current shifts perpendicular to the aromatic plane have never been subjected to anything more than qualitative verification. The presence of the five-membered heterocyclic imidazole ring of histidine and the indole ring of tryptophan present difficulties for any relatively simple molecular orbital approach to ring current fields associated with these residues. For these reasons, analyses of the pmr spectra of lysozyme and cytochrome *c* to be discussed subsequently in this paper will be qualitative in nature. It is expected, however, that studies in progress involving perturbations of spectra by inhibitor binding to lysozyme and amino acid substitutions in cytochrome *c* from diverse species will lead to more specific assignments.

As discussed above, observed high-field shifts of up to 1.4 ppm for the methyl and methylene groups of side chains of some of the component amino acid residues of lysozyme in the folded conformation are compatible with their originating from ring current effects induced by proximity of these groups to the faces of the aromatic rings of component histidine, phenylalanine, tyrosine, and tyrptophan residues. Expanded versions of the high-field spectral regions of lysozyme in the denatured (bottom) and folded (top) conformations are shown in Figure 8. The six valine, eight leucine, and six isoleucine residues are expected to contribute a total of 128 protons to the intensity of the 0.85-ppm resonance of the 78° spectrum. Estimates, based on area measurements, of the numbers of protons contributing to the various peaks of the 0.7- to −0.7-ppm region of the native conformation of lysozyme (the 64° spectrum of Figure 8) are as follows: at −0.68 ppm, 4.4 protons; the group of two weak and one strong resonances from −0.30 to 0 ppm, 23 protons; at 0.32 ppm, 11 protons; at 0.5 ppm, 10 protons. Thus, it appears that the resonances of about 48 protons are shifted from positions of lower shielding to the 0.7- to −0.7- ppm region when the folded conformation of the protein is assumed. The 128 protons of leucine, isoleucine, and valine of folded lysozyme contribute part but not all of the intensity to this high-field region.

Assignment of the various high-field resonances to specific proton–aromatic ring current interactions is impossible on an *a priori* basis. The three-dimensional structure of crystalline egg-white lysozyme is, however, known in considerable detail from the elegant X-ray studies of Phillips and co-workers.[24,25] From a model of lysozyme based on the X-ray coordinates[26] it was possible to identify sufficiently close approaches of the more shielded methyl and methylene groups of certain of the side chains of component residues to the faces of the aromatic rings of the component aromatic amino acids that could produce the high-field ring current shifted resonances observed in the folded conformation of lysozyme. These interactions are listed in Table II.[27] For reasons given above, it must however be emphasized that any such assignment must for the present be regarded as tentative. A basis for more defi-

(23) For a critical discussion of ring current effects in nmr, see J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," Vol. 1, Pergamon Press Inc., New York, N. Y., 1965, pp 140–149.

(24) C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature*, 206, 755 (1965).

(25) D. C. Phillips, *Sci. Am.*, 215, 78 (1966).

(26) Constructed on the basis of the X-ray coordinates in the laboratory of Dr. C. B. Anfinsen and kindly made available to us for inspection by the latter.

(27) A similar analysis of the high-field region of the 220-Mcps pmr spectrum of lysozyme has been carried out by H. Sternlicht (private communication).

nite assignments will perhaps be provided by studies in progress that reveal highly specific perturbations on the spectrum of lysozyme that result from interaction in solution between the enzyme and an inhibitor, the trimer of N-acetylglucosamine. Indeed, preliminary studies reveal that the resonances in the −0.30- to 0-ppm region are the only ones in the 0.7- to −0.7-ppm region affected by enzyme–inhibitor interaction. X-Ray studies by Phillips and co-workers[28] have shown that tryptophan residues 62, 63, and 108 are involved in binding the trimer and in fact undergo small relative displacements as the result of the interaction. Also, from Table II it is seen that certain side chains are sufficiently near these three tryptophan residues that conformational perturbations at these sites should result in altered ring current shifts. As might be expected, the aromatic region of the pmr spectrum of lysozyme is also affected as the result of the binding of the inhibitor. This result can be taken to indicate that pmr may rather sensitively reflect conformational perturbations in enzymes caused by substrate, inhibitor, and effector interactions.

Table II. Possible Interactions Leading to High-Field Resonances of Folded Lysozyme[a]

Aromatic residue	Residue in diamagnetic ring current field
Phe 3	Leu 8; Ileu 55; Lys 1 (Thr 40; Ileu 88)
His 15	Val 92 (Arg 14; Leu 17)
Tyr 20	Lys 96; Arg 21
Tyr 23	Met 105
Trp 28	Met 105; Leu 17
Phe 34	Arg 114 (Lys 33)
Phe 38	Lys 33 (Arg 5)
Tyr 53	(Thr 51; Thr 43; Leu 84)
Trp 62	Arg 61
Trp 63	Ileu 98; Leu 75
Trp 108	Ala 107 (Ileu 98; Val 99)
Trp 111	(Met 105)
Trp 123	(Val 29)

[a] Abbreviations used are: Phe, phenylalanine; His, histidine; Tyr, tyrosine; Trp, tryptophan; Lys, lysine; Ileu, isoleucine; Val, valine; Leu, leucine; Thr, threonine; Arg, arginine; Met, methionine; Ala, alanine. Residue in parentheses where weak interaction is expected.

In light of the above analysis of the high-field resonances observed in the 0.7- to −0.7-ppm region of lysozyme in the folded conformation, it is of interest to return briefly to ribonuclease. High-field resonances in this region were not observed in the pmr spectrum of native ribonuclease (Figure 2). Recently a great deal of progress has been made in elucidating by X-ray spectroscopy the three-dimensional structure of this enzyme.[29,30] Inspection of a three-dimensional model[31] based on the results of the Carlisle, Harker, and Richards groups indicates that highly shielded methyl and methylene groups are not well positioned with respect to the faces of the aromatic rings of the histidine, phenylalanine, and tyrosine residues to experience high-field ring current shifts in the folded conformation of the protein. In this respect, the pmr results and the emerging detailed X-ray structure of native ribonuclease appear to be in qualitative agreement.

Cytochrome *c*. Cytochrome *c* is an iron-containing protein that is involved in the respiratory cycles of plants and animals.[32] Horse heart cytochrome *c*, a typical mammalian representative of the class of proteins, consists of a single polypeptide chain of 104 amino acids with a molecular weight of 12,363. The single iron atom of the protein is present as part of a heme group and its facile interconversion between ferric and ferrous oxidation states is an important factor in its biological redox role. The heme group is bound to the polypeptide chain by means of two thioether linkages that involve, in the case of horse heart cytochrome *c*, two cysteine residues at the 14th and 17th amino acid positions of the chain. It is believed that the 5th and 6th coordination sites of the heme iron atom are occupied by donor constituents of basic amino acid side chains. Imidazole groups of two of the three histidine residues of the molecule long have been implicated as such ligands.[32] It has also been suggested that the two out-of-plane coordination sites of the heme iron were occupied by an imidazole of histidine and an ε-amino group of lysine.[33] However, recent X-ray results on oxidized horse heart cytochrome *c* appear to have established conclusively that only one histidine is coordinatively bound to iron.[34] It also appears quite probable that the groups bound to iron in the oxidized and reduced forms of cytochrome *c* are not identical.[35]

We wish to report here in preliminary fashion some 220-Mcps pmr characteristics of horse heart cytochrome *c*. Some of the results were derived from spectra obtained in 5 *M* urea. Urea is a classical denaturant and was employed in these initial studies to lower the denaturation temperature and, consequently, to confer a greater time–temperature stability on the samples in the denatured form. Subsequent studies in the absence of urea have yielded similar protein denaturation characteristics, but at somewhat elevated temperatures.

The temperature dependence of the pmr spectrum of horse heart ferricytochrome *c* is shown in Figure 9. The strong resonances of 4.6 and 5.7 ppm arise, respectively, from nonexchanged protons of water and urea. The spectrum is only partial in the sense that in the fully folded conformation of the protein there are resonances that extend all the way to 2.5 ppm to high field of the reference. These resonances will be discussed in some detail later in this section.

The aromatic amino acids of horse heart cytochrome *c* consist of three histidine, four phenylalanine, four tyrosine, and one tryptophan residues. In the denatured form of the protein (the 65° spectrum of Figure 9), the resonance absorption of cytochrome *c* in the aromatic region (6.5–7.5 ppm) is comparatively simple and can be assigned in relatively straightforward fashion. The peak at 6.75 ppm arises from *o*-phenyl protons and that at 7.1 ppm from *m*-phenyl protons of tyrosine;

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(31) Kindly made available to us for inspection in the laboratory of F. M. Richards.
(32) For an excellent, recent review of cytochrome *c*, see E. Margoliash and A. Schejter, *Advan. Protein Chem.*, 21, 114 (1966).
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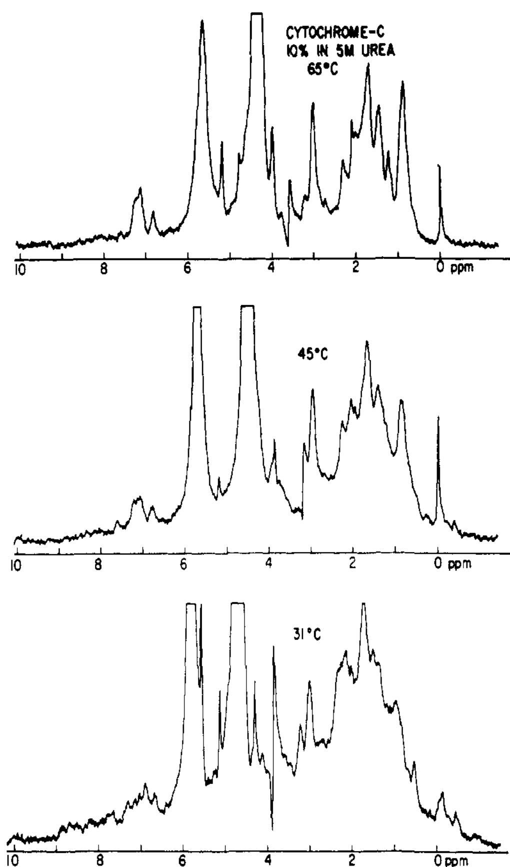

Figure 9. Temperature dependence of the 220-Mcps pmr spectrum of horse heart cytochrome *c*.

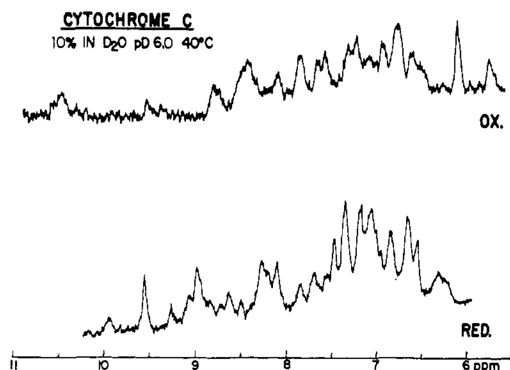

Figure 10. The "aromatic" regions of the pmr spectra of the oxidized and reduced forms of native horse heart cytochrome *c* at 220 Mcps.

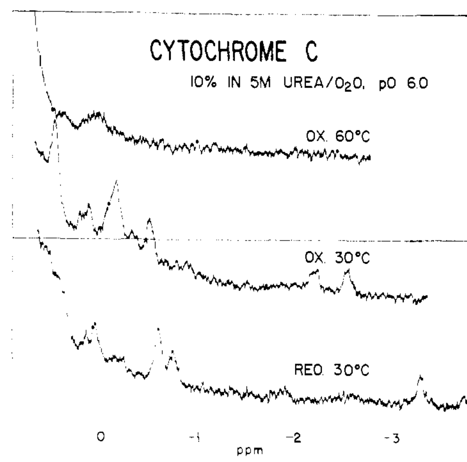

Figure 11. Radiofrequency absorption at 220 Mcps in the high-field region of the pmr spectrum of horse heart cytochrome *c*: top, denatured ferricytochrome *c*; middle, native ferricytochrome *c*; bottom, native ferrocytochrome *c*.

the aromatic protons of phenylalanine appear as the fairly broad peak centered at 7.2 ppm; and the C-2 protons of histidine yield the weak resonance at 7.6 ppm. Again, referring to the denatured form of the protein, the strong, well-resolved peak to highest field (0.85 ppm) is attributable to the six methyl protons of each of the valine, leucine, and isoleucine residues of which there are a total of 15 in horse heart cytochrome *c*. Of the 104 amino acid residues in cytochrome *c*, 19 are lysine. Side-chain CH_2 protons of lysine contribute to the two sharp resonances that are prominent in the 65° spectrum at 1.6 and 2.9 ppm.

Of particular interest are spectral changes that reflect the folding of the molecule into its biologically active conformation. As the temperature of the solution is reduced, the resonance absorption of the aromatic protons of the protein becomes very complex. This complexity is illustrated in Figure 10 in the expanded versions of the pmr spectra of the aromatic protons of ferricytochrome *c* (top) and ferrocytochrome *c* (bottom) in the native conformation. The very great differences between the pmr spectra of the oxidized and reduced forms of the molecule that are observed in the region of aromatic absorption are, as we shall see, to be found in other regions of the spectrum as well.

There is no pmr absorption in the high-field region of the spectrum beyond 0.85 ppm in the denatured forms of either the ferricytochrome *c* or the ferrocytochrome *c*. However, as the protein reassumes its native conformation as the temperature is reduced to below 60° the 0.85-ppm resonance which has been attributed to the highly shielded methyl groups of the valine, leucine, and isoleucine residues decreases in intensity and is partially replaced by a series of resonances that extend, in the case of the ferrocytochrome *c*, 4.5 ppm to even *higher* field (Figure 11). The high-field satellite resonances of both the oxidized and reduced forms of cytochrome *c* that are observed in the region +0.5 ppm to −1.0 ppm appear to be similar in origin to those observed earlier in lysozyme, namely, high-field ring current shifted resonances that arise from close proximity in the folded conformation of the protein of highly shielded C–H side-chain protons to the faces of the aromatic rings of phenylalanine, tyrosine, tryptophan, and histidine. Ring current shifts of 1.5 ppm are compatible with the positioning of the protons of, say, a methyl group along the sixfold axis of a benzene ring and at a distance of about 3.2 A above the plane of the ring, by no means an impossible packing geometry. The observed shifts in the two forms in the 3.0–4.5-ppm range are more difficult to account for on this basis. A separation of only 2.1 A between methyl protons and the plane of a benzene ring is required for a ring current shift of 4.2 ppm, an unreasonably small distance.

There would appear to be two possible mechanisms for producing the high-field satellite resonances that occur in the −2.0- to −3.8-ppm region of the pmr spectrum of the oxidized form of cytochrome *c* in the

folded conformation. The first of these, contact shifts,[36] either isotropic hyperfine or pseudo-contact, is rejected on the following counts. Contact shifts, probably of the isotropic hyperfine variety, were earlier observed by Kowalsky[7] for ferricytochrome *c* in the folded conformation and have been verified by us. These severely broadened contact shifted resonances appear at about +25 ppm to low field and −33 ppm to high field. The high-field satellite resonances of cytochrome *c* in which we are presently interested are observed for not only the paramagnetic ferricytochrome *c* (−2.1 to −2.5 ppm) but for the diamagnetic ferrocytochrome *c* (−3.2 to −3.8 ppm) as well. Thus, paramagnetism, a necessary prerequisite to contact shifts of any variety, cannot be responsible for the high-field satellite resonances of ferrocytochrome *c* which are, in fact, greater than those observed in the paramagnetic ferricytochrome *c*. Another, perhaps even more decisive, argument is that the line widths of the −2.1- to −2.5-ppm satellite resonances of ferricytochrome *c* are only about 20 cps, about the same as those of the resonances of the −3.2- to −3.8-ppm region of ferrocytochrome *c* and at least as narrow as the previously discussed high-field satellite resonances of lysozyme. Contact shifted resonances customarily are broadened through electron–nucleus dipolar interaction.[36] Thus, it appears fairly certain that the high-field satellite resonances in the −2.1- to −3.8-ppm regions of the spectra of the ferricytochrome *c* as well as the ferrocytochrome *c* are produced by a mechanism other than contact interaction.

It is likely that the high-field resonances of the −2.1- to −3.8-ppm regions of the oxidized and reduced forms of cytochrome *c* arise from ring current shifts of side chains of component valine, leucine, and isoleucine residues that reside in the folded conformation in close proximity to the faces of the porphyrin *c* moiety. Previous workers have shown that huge ring current shifts of 10 ppm or more can be associated with the extensively delocalized porphyrin ring.[21,22] Unfortunately, and for reasons discussed earlier, it is not presently possible to make any meaningful comparison between theoretical phorphyrin ring current shifts and those observed experimentally in cytochrome *c*. Nevertheless, it is suggested that this effect is responsible for the −2.1- to −3.8-ppm shifts observed in the pmr spectra of the oxidized and reduced forms of horse heart cytochrome *c*.

As noted earlier, differences exist in the aromatic regions of pmr absorption for ferrocytochrome *c* and ferricytochrome *c* in their folded conformations. As is evident from Figure 11, these differences extend most strikingly into the region to high field of the methyl resonances of the leucine, isoleucine, and valine residues of the denatured protein. It would appear then that the folded conformations of the ferricytochrome *c* and ferrocytochrome *c* are significantly different. Another piece of evidence compatible with this hypothesis is that the ferrocytochrome *c* is considerably more stable to thermal denaturation than ferricytochrome *c*; in 5 *M* urea the former is stable to about 85°, while the latter thermally denatures at 65°. The X-ray evidence of Dickerson[34] seems to be conclusive in assigning the 5th

(36) For a review of contact shifts, see D. R. Eaton and W. D. Phillips, *Advan. Magnetic Resonance*, 1, 103 (1965).

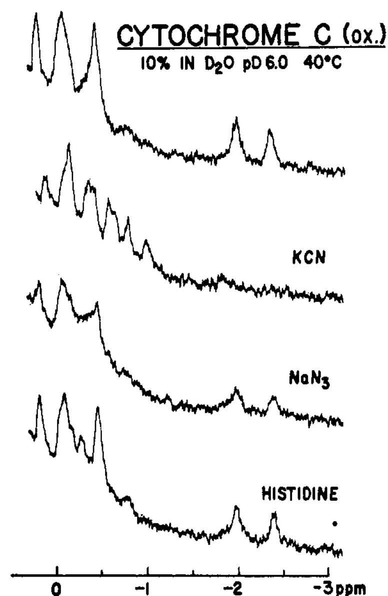

Figure 12. Effect of cyanide coordination on the high-field pmr absorption of ferricytochrome *c*. Under the experimental conditions employed, coordination between the heme iron and added azide ion and histidine did not occur.

and 6th coordination sites of the iron of ferricytochrome *c* to histidine and some other residue, perhaps methionine. Some alteration in coordination at at least one of these sites appears required for ferrocytochrome *c*. The profound effect of altered iron coordination on the pmr spectrum of ferricytochrome *c* is shown in Figure 12. Incubation of the protein with azide ion and histidine has little or no effect on the spectrum. Cyanide ion, however, which apparently displaces one or both of the amino acid side-chain ligands at the 5th and 6th coordination sites, profoundly alters the spectrum of ferricytochrome *c* in the high-field satellite region. As a matter of fact, the two high-field resonances at −2.1 to −2.4 ppm of the ferricytochrome *c* spectrum that were attributed to ring current shifts arising from proximity of methyl groups to the porphyrin ring are missing in the cyano derivative. Equally extensive changes in the pmr spectrum of the aromatic region of ferricytochrome *c* are produced as the result of coordination with cyanide.

Temperature dependences in the high-field satellite region of the oxidized and reduced forms of cytochrome *c* are shown in Figures 13 and 14. The integrated intensity of the single sharp resonance that occurs at −3.2 ppm in the spectrum of ferrocytochrome *c* is consistent with its arising from a single CH_3 group. The three clearly resolved peaks centered around −0.45 ppm in the 50° spectrum of ferrocytohrome *c* similarly are attributable, on an intensity basis, to methyl groups. It is noteworthy, particularly in the case of the ferrocytochrome *c* (Figure 14), that the positions of the high-field satellite resonances at temperatures considerably below thermal denaturation exhibit a slight temperature dependence. Temperature dependences over this range in the region of aromatic resonance absorption for both the ferrocytochrome *c* and ferricytochrome *c* are even more striking. It is thus clear, and perhaps not surprising, that the conformation of cytochrome *c*, as

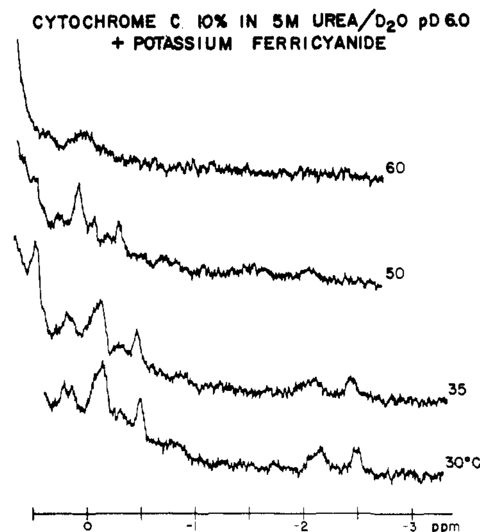

Figure 13. Temperature dependence of the high-field region of the 220-Mcps pmr spectrum of ferricytochrome *c*.

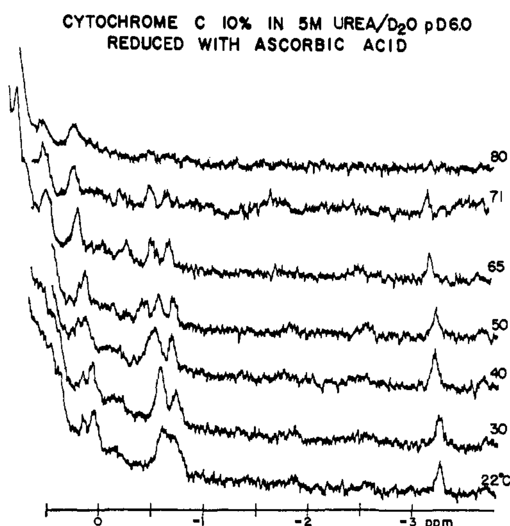

Figure 14. Temperature dependence of the high-field region of the 220-Mcps pmr spectrum of ferrocytochrome *c*.

probably with the majority of proteins, is continuously altered with changes in environmental conditions.

Oxidation–reduction equilibria in cytochrome *c* systems can be followed by electronic spectral absorptions characteristic of the two forms[37,38] and, with more difficulty, by magnetic susceptibilities.[39] It is apparent from the pmr spectrum of a partially reduced sample of ferricytochrome *c* (Figure 15) that pmr also can be employed to monitor such equilibria. Resonances attributable to ferricytochrome *c* and ferrocytochrome *c* are cleanly resolved and the redox equilibrium can be determined from appropriate integrated resonance intensities.

The pmr spectra of myoglobin and hemoglobin are being investigated and will be detailed in a subsequent publication. It is, however, worth noting in connection with the above discussion of cytochrome *c* that extreme high-field resonances in the −2.1- to −3.8-ppm region

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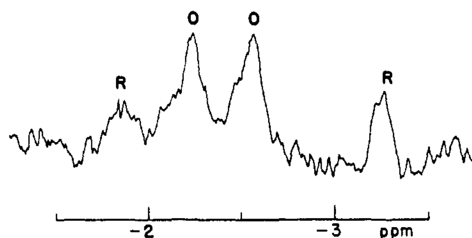

Figure 15. Partial spectrum of the high-field region of a mixture containing both oxidized, O, and reduced, R, forms of horse heart cytochrome *c*.

were not observed for the folded conformations of either of these proteins in solution under normal spectrometer operating conditions. Two explanations for the absence of such resonances in myoglobin and hemoglobin seem possible. One is that the side chains of none of the more shielded C–H protons of side chains are sufficiently close to the faces of the porphyrin ring to experience sizable high-field ring current shifts into the −2.1- to −3.8-ppm region. Although the heme groups of myoglobin and hemoglobin appear to be much nearer the surfaces of the molecules and more exposed to the exterior than that of cytochrome *c*, inspection of a model of whale myoglobin[26] suggests that the methyl groups of, at the very least, the valine-68, leucine-89, and isoleucine-99 residues should be favorably situated with respect to the faces of the porphyrin ring to be subject to appreciable ring current field interactions. It thus appeared that this explanation for the apparent absence of resonances in the −2.2- to −3.8-ppm region of the pmr spectra of myoglobin and probably hemoglobin as well could be dismissed, at least tentatively. However, it seemed possible that resonances from the side-chain methyl groups of these amino acids were present in this region of resonance absorption but were severely broadened through nucleus–electron dipolar interaction by the sizable magnetic moments of the iron of myoglobin and hemoglobin. The heme iron of these proteins, in contrast to that of cytochrome *c*, is high spin with moments in the ferri form of 5.9 BM and in the ferro form of 4.9 BM. The effectiveness of a paramagnetic center in broadening a nuclear resonance varies as the square of the paramagnetic moment and the inverse sixth power of the separation between the nucleus and the center. Then, for comparable nucleus–electron distances, the iron of myoglobin and hemoglobin should be six to eight times more effective than that of ferricytochrome *c* in relaxing vicinal protons. Paramagnetic relaxation should not of course be a factor in determining the line widths of resonances of the diamagnetic ferrocytochrome *c*.

The pmr spectra of the cyano-coordinated derivatives of myoglobin and hemoglobin would be expected to be of value in elucidating this point since in these derivatives the iron is converted to the low spin case encountered in cytochrome *c* where nucleus–electron dipolar broadening effects should be and appear to be much less extensive. Indeed, the cyano derivative of myoglobin exhibits two new resonances, each displaying an intensity equivalent to one methyl group, in the −3.0- to −3.3-ppm region of the pmr spectrum. This observa-

tion prompted reexamination of the −2.2- to −3.8-ppm region of the spectrum of native myoglobin. With 250 computer-averaged traces, a resonance of 110-cps half-maximum width emerged at −3.72 ppm. These results would seem to justify the conclusion that side-chain groups positioned sufficiently near to the porphyrin ring to experience sizable ring current shifts are present in myoglobin and probably hemoglobin as well as in the previously discussed cytochrome *c*. It would appear that such resonances of high-spin myoglobin and hemoglobin are broadened because of dipolar relaxation by the heme iron, although the effect alternatively could originate in differences in electronic relaxation times of iron in the reduced and oxidized forms of the protein.

Acknowledgments. We are grateful to Dr. R. C. Ferguson for advice and assistance in the maintenance and operation of the 220-Mcps spectrometer and to Messrs. F. W. Barney and F. V. Ferrari for technical assistance in obtaining the spectra.

The Metabolic Products of Naphthalene in Mammalian Systems[1]

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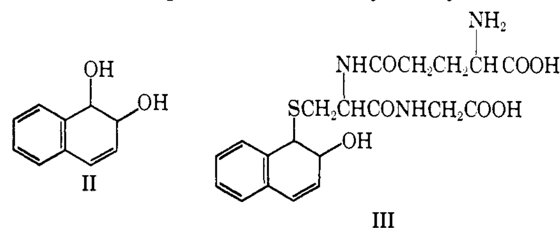
Received July 31, 1967

Abstract: The enzymatic conversion of naphthalene to 1,2-dihydro-1,2-dihydroxynaphthalene has been shown by labeling experiments to proceed with the incorporation of one atom of oxygen from molecular oxygen, the second oxygen atom being derived from water. The initial attack upon the substrate takes place at the α position and the product has been shown by nmr spectroscopy to be the *trans* diequatorial diol. The over-all mechanism of the oxygenation is discussed in the light of these findings.

In 1955 it was demonstrated independently by Hayaishi, *et al.*,[2] and by Mason, *et al.*,[3] that molecular oxygen may be directly incorporated into various substrates. Such reactions are now generally termed oxygenations to distinguish them from oxidations, in which molecular oxygen is merely an electron acceptor. It is now quite clear that oxygenases, the enzymes that catalyze oxygenations, are of great importance in that they are involved in the metabolism of a great variety of substrates such as steroids,[4] aromatic hydrocarbons,[5] alkaloids,[6] and numerous drugs[7] such as aminopyrine[8] and the barbiturate, seconal.[9] The metabolism of naphthalene in mammalian systems[10] has been thought[11] to proceed *via* an oxygenation step, the mechanism of which is, however, obscure. As this substrate is representative of the polycyclic carcinogens, it was deemed of interest to study the pathway by which it is oxidatively metabolized.

Among the major metabolites of naphthalene in rabbits and rats are α-naphthol (I), 1,2-dihydro-1,2-dihydroxynaphthalene (DHN-diol, II),[12,13] and S-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione (III). Further work on *in vitro* systems showed that formation of I and II from naphthalene is catalyzed by liver microsomal enzymes which require NADPH and molecular oxygen.[14] It has not been possible to classify these enzymes as oxygenases since it has not been demonstrated that molecular oxygen is directly incorporated into naphthalene. A molecule of oxygen may be incorporated intact, the enzyme involved being a dioxygenase, or it may donate one atom of oxygen, the enzyme catalyzing such a reaction being a monooxygenase of the mixed function type,[15] since NADPH is required. It is known[14,16] that neither I nor II may serve as a precursor of the other, but whether they both have a common precursor is unclear.



We have already adduced evidence[1] showing that only one atom of molecular oxygen is incorporated into naphthalene in the formation of II and now wish to report experiments which show that naphthalene is converted by a monooxygenase to both I and II, possibly *via* a common precursor.

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