Effects of Chemical Modification and $CoCl_2$ Addition on the ^{13}C NMR Spectra of Carnosine as a Solid Powder

Charles Eric Brown

Contribution from the Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226. *Received May 27, 1980*

Abstract: The ^{13}C NMR spectra of the dipeptides carnosine, anserine, homocarnosine, and glycyl-L-histidine and component amino acids in solid powders are compared. The observed chemical shifts are the same as would be expected from high-resolution NMR spectroscopy, but the observed sensitivity is very dependent on the identity of the N-terminal amino acid residue. Signal intensities from carnosine are greatly enhanced by addition of $CoCl_2$. The sensitivity of ^{13}C NMR spectra recorded with cross polarization and magic angle sample spinning may be improved without seriously affecting resolution by introduction of paramagnetic metal ions or free radicals.

Comparison of ^{13}C NMR spectra of benzenoid and heterocyclic analogues of poly(*p*-phenylene) and lower oligomers that are recorded with cross polarization and magic angle sample spinning (CP/MAS) indicates that chemical structure greatly affects the intensity of NMR spectra of solids.[1,2] The intensities of CP/MAS ^{13}C NMR spectra of poly(*p*-phenylene) and its lower oligomers were found to increase as the free-radical contents of these oligomers increase, but the resolution appeared not to be seriously affected.[1,2] This observation suggests that the sensitivity of ^{13}C NMR spectroscopy to solid samples might also be increased by the introduction of paramagnetic metal ions.

Carnosine (β-alanyl-L-histidine) provides a well-characterized sample for determining the effect of paramagnetic metal ions on CP/MAS ^{13}C NMR spectra. The structure of carnosine in the crystal has been determined[3,4] and compared with that in aqueous solution as determined with high-resolution ^1H NMR spectroscopy.[3,5,6] Furthermore, the structures of the metal chelates of carnosine have been well characterized under a variety of conditions.[7-17] In this paper, I compare the CP/MAS spectra of carnosine and its biologically active analogues and demonstrate the value of paramagnetic doping in recording high-resolution ^{13}C NMR spectra of carnosine in the solid state.

Experimental Section

^{13}C NMR spectra of solid samples were recorded with a Nicolet NT-150 spectrometer operating at 37.7 MHz. Cross polarization[18,19] and proton decoupling were used to record these spectra. Samples were spun at the magic angle[20] in a Delrin rotor with a cylindrical sample chamber 12 mm long and 10 mm in diameter. All samples filled the chamber except anserine nitrate (Figure 1E), which only half-filled the rotor. Spinning frequencies were estimated from the locations of spinning sidebands arising from the rotor and/or the sample. Spectra were recorded at two different spinning rates to distinguish resonances from spinning sidebands. The spectra presented in Figures 1 and 2 were recorded at spinning frequencies of about 2.0-2.4 kHz.

The Delrin rotor yields an intense ^{13}C resonance at 90 ppm relative to Me_4Si and a much weaker resonance at 69 ppm. Since the intensities of these resonances are not seriously affected by extending the delay between pulses beyond 1 s or by doping the sample with low levels of paramagnetic metal ions, the rotor can be used as an internal standard for ^{13}C signal intensities. The wide range of signal intensities from the various samples required that the ^{13}C spectra in Figures 1 and 2 be presented with the most intense signal from each sample having the same height. Thus, an increase in signal intensity from the sample is observed as a reduction in the noise level and a reduction in intensity of the resonances and spinning sidebands from the rotor. Signal-to-noise ratios and relative signal intensities of the spectra in Figure 2 are presented in Table I.

Table I. Signal-to-Noise Ratios and Signal Intensities Relative to an Internal Standard Measured from Absolute Intensity Plots of the Spectra in Figure 2

spectrum	S/N[a]	peak intensity relative to an internal standard[b]
(A) cobalt-carnosine; 1-s delay	38.8	0.19
(B) carnosine; 5-s delay	22.0	0.11
(C) carnosine; 3-s delay	16.9	0.10
(D) carnosine; 1-s delay	10.0	0.06

[a] The signal-to-noise ratio for the resonance of the carboxyl group (180 ppm) was calculated with the Nicolet software. [b] This is the ratio of the signal intensity of the carboxyl group resonance (180 ppm) divided by the intensity of the Delrin resonance (90 ppm).

Results

CP/MAS ^{13}C NMR Spectra of Carnosine and Its Analogues. Carnosine (β-alanyl-L-histidine) as a solid powder exhibits ^{13}C resonances at about the same chemical shifts (Figure 1A) as does carnosine in aqueous solution (Figure 1B). However, the nonprotonated carbons give rise to the most intense resonance in the solid, whereas the protonated carbons do so in solution. This is similar to our observation with analogues of poly(*p*-phenylene).[1,2]

Compared with the resonances from the rotor (90 ppm and 69 ppm, labeled R), carnosine does not yield a very intense NMR spectrum as a solid (Figure 1A). The same is true for homo-

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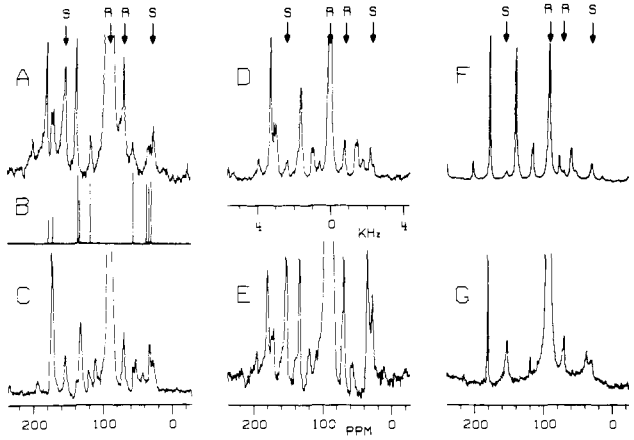

Figure 1. CP/MAS ^{13}C NMR spectra of solid (A) carnosine, (C) homocarnosine sulfate, (D) glycyl-L-histidine hydrochloride, (E) anserine nitrate, (F) L-histidine, and (G) β-alanine; and (B) the high-resolution ^{13}C NMR spectrum of carnosine dissolved in D_2O, pH 7.2. The number of acquisitions are (A) 15000, (C) 10000, (D) 2000, (E) 15000, (F) 5000, and (G) 5000. All spectra were recorded with a delay between pulses of 1 s. The assignments of these spectra are as follows:[23] carboxyl groups of histidyl residue, ca. 180 ppm; peptide carbonyl of β-alanyl residue, ca. 172 ppm; C-2 and C-5 of the imidazole ring, 138 ppm; C-4 of the imidazole ring, 117 ppm; α-carbon atom of the histidyl residue, 57 ppm; and the methylene groups of the histidyl and β-alanyl residues, 31–35 ppm. The resonances from the Delrin rotor at 90 and 69 ppm are labeled R, and the first-order spinning sidebands from the resonance at 90 ppm are labeled S. Spinning sidebands from the sample also are apparent in (C).

carnosine (γ-aminobutyryl-L-histidine, Figure 1C), glycyl-L-histidine (Figure 1D), anserine (β-alanyl-1-methylhistidine, Figure 1E) and β-alanine (Figure 1G), although there is some variation in signal intensity among them. For example, glycyl-L-histidine (Figure 1D) yields a signal-to-noise ratio 5–6 times greater than that observed with carnosine under the same conditions (Table I). In contrast, L-histidine yields a very intense CP/MAS ^{13}C NMR spectrum (Figure 1F) compared to those of the peptides of which it is a part. For example, the intensity of the carboxyl resonance of histidine is comparable to that of the Delrin rotor (Figure 1F), whereas the intensity of the carboxyl resonance of carnosine is only about 6% of that of the rotor with the same delay between pulses (Figure 1A, Figure 2D, and Table ID).

In other studies we observed that the CP/MAS ^{13}C NMR signal intensities from poly(*p*-phenylene) and its lower oligomers are related to their free-radical content[1] and that oligomers with low concentrations of free radicals yield CP/MAS ^{13}C spectra only with long delays between pulses. By analogy, we reasoned that the low intensity of the carnosine spectrum might arise from long proton T_1 values and that doping with a paramagnetic metal ion might increase the apparent sensitivity by increasing the rate of relaxation of the protons to which the ^{13}C nuclei are cross polarized. Therefore, CP/MAS spectra were recorded (i) with increasing delays between pulses and (ii) after doping with cobalt(II) chloride under conditions that yield only partial oxidation of the cobalt.[14] It was found that within this family of very similar compounds, which vary by as little as a methylene group, the T_1 values of the protons vary at least 5-fold (for example, Figures 1 and 2). Furthermore, doping with cobalt permitted a shorter delay between pulses without loss of sensitivity and had little effect on the observed resolution (Figure 2A). On the basis of signal-to-noise ratios and comparison of signal intensities to an internal standard, doping with $CoCl_2$ produces a 3–4-fold increase in the intensity of the carnosine spectrum recorded with a delay between pulses of 1 s (Table I, A and D).

It should be noted that all of the ^{13}C resonances in the solid increase in intensity to the same extent upon extending the delay between pulses (Figure 2B–D). This is quite different from the results obtained with solutions, in which the nuclei with the longest T_1 values are the most affected by too rapid a repetition rate. Furthermore, doping with a paramagnetic metal ion also increases

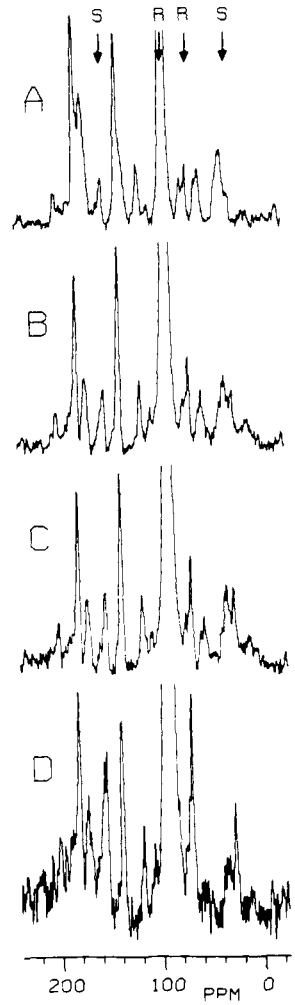

Figure 2. CP/MAS ^{13}C NMR spectra of (A) solid cobalt–carnosine (1:10 molar ratio) and (B–D) carnosine. All spectra were obtained with 2000 acquisitions. The delays between pulses are (A and D) 1 s, (B) 5 s, and (C) 3 s. The resonances from the Delrin rotor at 90 and 69 ppm are labeled R, and the first-order spinning sidebands from the resonance at 90 ppm are labeled S.

the intensity of all the ^{13}C resonances to the same extent in the solid, whereas the protons of carnosine that are nearest to the site of ligation are most affected by paramagnetic metal ions in solution.[10,13–17]

Discussion

The crystal structure of carnosine is ordered with the carnosine molecule in a conformation similar to that observed in solution but more restricted.[3,4] The acid and basic groups are engaged in hydrogen bonds,[3] and this hydrogen bonding is expected to maintain the order in the crystal and to reduce the freedom of molecular motion. Thus chemical shift distributions, which can give rise to multiple peaks in the spectra of disordered solids, are not expected with carnosine and, indeed, are not apparent. The CP/MAS ^{13}C NMR spectrum of solid carnosine exhibits peaks at the same chemical shifts (Figure 1A) as are observed for carnosine in aqueous solution (Figure 1B). However, the CP/MAS ^{13}C spectrum of carnosine is of very low intensity and thus is difficult to record. Long delays are required between pulses to obtain acceptable signal to noise. In sharp contrast, histidine, one of the two component amino acids of carnosine, yields a CP/MAS ^{13}C spectrum about 20-times more intense than that of carnosine, with a delay between pulses of only 1 s.

The relaxation rates of protons in solids can vary to a large extent with only minor variations in chemical structure. Thus cross polarization, which relies on the relaxation of protons to observe dilute spins, can be seriously hampered by slow ^1H relaxation rates. This appears to be the reason for the observed

differences in signal intensities among the various dipeptides and amino acids investigated here. Hydrogen bonding within the carnosine crystal is expected to restrict the freedom of molecular motion and thus to produce longer T_1 values than those observed with more mobile solids.[21]

Since small amounts of paramagnetic centers can increase the relaxation rates of the protons without affecting the line widths of the ^{13}C resonances,[1] the sensitivity of CP/MAS NMR spectra of solids that exhibit slow relaxation can be improved by paramagnetic doping. This observation was confirmed independently after submission of this manuscript.[22] Because there is a high level of dipolar coupling between protons in such solids and each ^{13}C nucleus can cross polarize to more than one proton, one might expect all the ^{13}C resonances to increase in intensity to about the same extent. This is indeed found. Although less than 40% of the carnosine molecules in Figure 2A are bonded directly to cobalt(II),[14,17] the peak intensities of the carnosine ^{13}C resonances are all affected equally. Thus addition of low concentrations of paramagnetic centers should be useful for increasing the sensitivity of CP/MAS ^{13}C NMR spectroscopy for solids with long proton T_1 values.

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Registry No. Carnosine, 305-84-0; homocarnosine sulfate, 19841-48-6; glycyl-L-histidine hydrochloride, 3486-76-8; anserine nitrate, 5937-77-9; L-histidine, 71-00-1; β-alanine, 107-95-9; cobalt chloride, 7646-79-9.

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Geometrical Structures of Electronically Excited States of Conjugated Hydrocarbons

T. Nakajima,*[1a] A. Toyota,[1b] and M. Kataoka[1a]

Contribution from the Department of Chemistry, Faculty of Science, Tohoku University, Sendai 980, Japan, and the Department of Chemistry, Faculty of General Education, Yamagata University, Yamagata 990, Japan. *Received December 2, 1981*

Abstract: On the basis of the pseudo Jahn-Teller theory, the geometrical structures with respect to C-C bond lengths of electronically excited states of conjugated hydrocarbons are examined. The Walsh-Pearson rule that the first excited state of a molecule should belong to the same point group as the ground state of its mono- or dinegative ion is insufficient for alternant hydrocarbons and does not hold for nonalternant hydrocarbons of a certain type. It is shown that in alternant hydrocarbons, the first excited singlet state does not undergo a molecular-symmetry reduction due to bond distortion, and in highly symmetrical alternant hydrocarbons such as benzene, the second excited singlet state suffers the same type of molecular-symmetry reduction due to bond distortion and, consequently, belongs to the same point group as the ground states not only of the mono- and dinegative ions but also of the mono- and dipositive ions. The first excited singlet states of pentafulvalene and nonafulvalene are predicted to belong to the same point group as the ground states of the respective positive ions.

On the basis of the pseudo, or second-order, Jahn-Teller effect and of molecular orbital (MO) theory, Pearson[2] has proposed a symmetry rule for predicting the geometrical structures of electronically excited molecules. The rule can be simply stated: the first excited state of a molecule containing n electrons should belong to the same point group as the ground state of a similar molecule having $n + 1$ or $n + 2$ electrons. The extra one or two electrons are assumed to be in that molecular orbital which becomes occupied in the excited state.

Exactly the same rule was given by Walsh[3] as early as 1953. His reasoning was based on the so-called Walsh diagram, in which the energies of the vacating MO and the filling MO are plotted against the change of nuclear positions.

The underlying assumption on which the Walsh-Pearson rule is based is that the promoted electron, being in a very unstable MO, is the one that is active in causing a structural change. The vacancy in the stable MO is presumed to be less active.

Pearson[2] has demonstrated how well the rule works for a variety of inorganic molecules, including transition-metal complexes and simple organic molecules. In this paper, we examine the applicability of the Walsh-Pearson rule to the prediction of the geometrical structures with respect to C-C bond lengths of the electronically excited states of conjugated hydrocarbons. It will be shown that the rule is insufficient for alternant hydrocarbons and does not hold for nonalternant hydrocarbons of a certain type.[4] It is revealed that the vacancy in the stable MO, which was ignored in deriving the Walsh-Pearson rule, is of primary importance in predicting the geometrical structures of excited states of pentafulvalene and nonafulvalene and is as important as the occupancy in an unstable MO in predicting those of alternant hydrocarbons.

Preliminary Theory

Assuming for a planar conjugated hydrocarbon the most symmetrical arrangement of C nuclei as the unperturbed system and using the second-order perturbation theory, we find that the ground-state energy after nuclear deformation by means of the C-C stretching normal mode Q_ν is given by[5]

$$E(Q_\nu) = E_0 + \left\langle \psi_0 \left| \frac{\partial H}{\partial Q_\nu} \right| \psi_0 \right\rangle Q_\nu + \frac{1}{2}\left\{ \left\langle \psi_0 \left| \frac{\partial^2 H}{\partial Q_\nu^2} \right| \psi_0 \right\rangle - 2\sum_{n\neq 0} \frac{\left| \left\langle \psi_n \left| \frac{\partial H}{\partial Q_\nu} \right| \psi_0 \right\rangle \right|^2}{E_n - E_0} \right\} Q_\nu^2 \quad (1)$$

If the initial ground state, ψ_0, is nondegenerate, as is the case with

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