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Natural Abundance ^{13}C Nuclear Magnetic Resonance Study of Gelatin†

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ABSTRACT: Natural abundance Fourier transform ^{13}C nuclear magnetic resonance (nmr) spectra were obtained for calf skin gelatin. Most resonances can be assigned to individual carbon atoms. Change of pH affects only the C^{γ} and C^{δ} resonances of the glutamic acid residues. Spin–lattice relaxation times were measured by the partially relaxed Fourier transform technique. The values are to be expected for a random coil molecule. The ^{13}C nmr spectra for poly(hydroxy-L-proline) are also reported.

Since the first ^{13}C nuclear magnetic resonance (nmr) study of amino acids by Horsley and Sternlicht (1968), the technique has been developed into a powerful new tool for elucidating the structures of biological molecules. A partial list includes studies of peptides (Horsley *et al.*, 1970), gramicidin S-A (Gibbons *et al.*, 1970), lysozyme (Chien and Brandts, 1971), chloresteryl chloride and AMP (Allerhand *et al.*, 1971), ribonuclease (Allerhand *et al.*, 1970; Glushko *et al.*, 1972), oligopeptides (Gurd *et al.*, 1971; Christl and Roberts, 1972), oligosaccharides (Dorman and Roberts, 1971), lecithin vesicles and erythrocyte membranes (Metcalfe *et al.*, 1971), carboxyhemoproteins (Conti and Paci, 1971; Moon and Richards, 1971), polynucleotides (Mantsch and Smith, 1972), and yeast transfer RNA (Komoroski and Allerhand, 1972). We have used ^{13}C nmr to study the helix–coil transition of collagen. In the course of this work, it was found necessary to thoroughly characterize the ^{13}C nmr of gelatin. In this paper, the nmr spectrum is reported along with the assignment of the individual carbon resonances, the effect of the pH, and the determination of spin–lattice relaxation times for the carbon nuclei in various residues.

Materials and Methods

Materials. Calf skin gelatin (NJ 869 0-69-792) was obtained from Kind and Knox Gelatin Co. The amino acid composition is taken to be that given by Veis (1964) for bovine corium collagen. It was dissolved at a concentration of 15 w/vol % in 0.2 M KCl, 0.2 M KH_2PO_4–K_2HPO_4 buffer, 2 M KCNS, 2 M $CaCl_2$, and 0.5 M $(NH_4)_2SO_4$. These solutions were then adjusted to the desired pH with either 1 N HCl or 1 N KOH using Radiometer type PHM 26 pH meter standardized at 25° for the measurement. Poly(hydroxy-L-proline) (mol wt 8120) was obtained from Sigma Chemical Co. and was dissolved in 6 M LiBr at a concentration of 0.28 M.

Methods. High-resolution ^{1}H broad band decoupled, natural abundance Fourier transform ^{13}C nmr spectra were were obtained at 22.63 MHz with a Brucker HFX-90 spectrometer. The ^{13}C analytical channel was operated with a 10-mm insert maintained at a temperature of 31° in the single coil mode where a 12-μsec pulse resulted in a $\pi/2$ spin nutation. Hexafluorobenzene contained in a 5-mm coaxial tube was used to secure field-frequency stabilization. Free induction decays were accumulated in a Nicolet 1080 time-averaging computer using 8192 channels internally swept at a rate of 100 μsec per channel (5 kHz frequency domain). Frequency components greater than 5 kHz were removed by a low-pass filter built into the Nicolet SD80 signal digitizer. A delay of 100 μsec was introduced between the time of the

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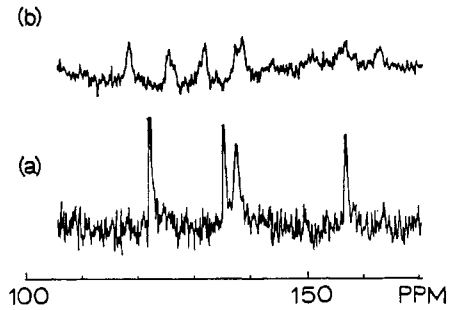

FIGURE 1: ^{13}C nmr spectra of poly(hydroxy-L-proline) of mol wt 8120 in 6 M LiBr, concentration 0.28 M: (a) ^{1}H-decoupled spectrum (12-μsec pulse, 1-sec recycle time, 16,384 transients); (b) ^{1}H-coupled spectrum (12-μsec pulse, 1-sec recycle time, 65,536 transients).

irradiating pulse and the time the receiver was first sampled by the Nicolet SW80 sweep generator. For the gelatin spectra, 16,384 transients were accumulated following a 10-μsec pulse with a pulse recycle time of 1 sec. The computer processing was performed utilizing the Nicolet Fourier transform package (NIC-80/S-7202f).

All resonances were found upfield from the CS_2 resonance, and chemical shifts are reported relative to CS_2 at 0 ppm. Those of poly(hydroxy-L-proline) were measured with respect to an internal dioxane reference (126.3 ppm).

Spin–lattice relaxation times were measured from partially relaxed Fourier transform spectra obtained from $(\pi-\tau-\pi)/2$ sequences. The sequence timing for the π and $\pi/2$ pulses as well as the trigger pulse for the time-averaging computer were provided by a Brucker MPG-2 four-pulse generator. For these measurements 16,384 transients were accumulated with a 3-sec sequence recycle time.

Values of T_1 were computed from the slope of a plot of ln $[(A_0 - A)/2A_0]$ *vs.* τ, where A is the resonance peak amplitude at the particular τ used, and A_0 is the amplitude of the completely recovered resonance.

Results and Discussion

^{13}C Nmr Spectra of Poly(hydroxy-L-proline). Hyp is an amino acid which is almost unique to collagenous proteins. Whereas ^{13}C nmr chemical shifts for most of the amino acids have been reported, those for Hyp have not. Poly(hydroxy-L-proline) is used in this work as the model compound for the hydroxy-L-prolyl residue in proteins.

The proton-decoupled ^{13}C nmr spectrum of poly(hydroxy-L-proline) is shown in Figure 1a; the proton-coupled spectrum is given in Figure 1b. The C^{α} resonance can be readily identified by its doublet feature in the coupled spectrum, where its chemical-shift value is 135.5 ppm, and $J_{C^{\alpha}H}$ is 150 Hz. Assignments of other resonances were based on the additivity rules of Grant and Paul (1964) as modified by Horsley and Sternlicht (1968) and Horsley *et al.* (1970) and comparison with the corresponding resonances in poly(L-proline) (Dorman *et al.*, 1973). The chemical-shift values for C^{β}, C^{γ}, and C^{δ} are 157.0, 122.7, and 137.6 ppm, respectively. The ^{13}C–H hyperfine coupling constants for C^{β}, C^{γ}, and C^{δ} are 140, 155, and 150 Hz, respectively.

The ^{13}C nmr spectrum of poly(hydroxy-L-proline), unlike that of poly(L-proline), where the two isomeric forms I and II become disordered in concentrated aqueous salt solutions (4.0 M $CaCl_2$ or 5 M KI), shows only one dominant isomer (Figure 1b) in salt solution (6 M LiBr) of comparable concentration. Apparently, the secondary structure of poly(hydroxy-L-proline) is stabilized by hydrogen bonding. X-Ray diffraction studies (Sasisekharan, 1959) showed that each chain is connected to its six neighbors by O—H···O=C

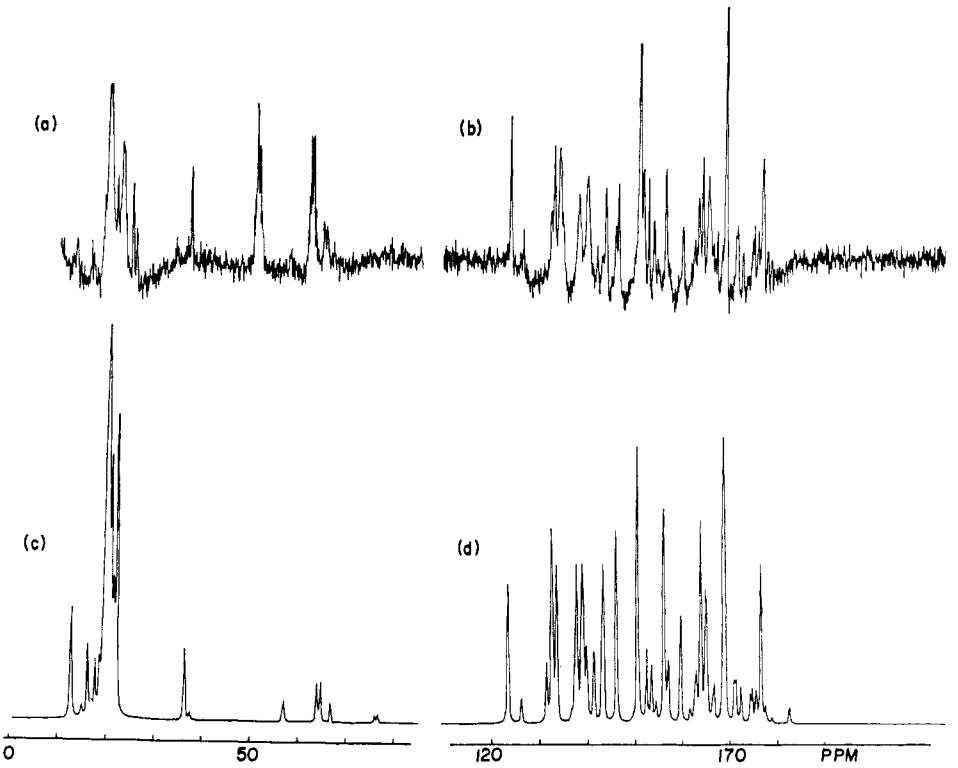

FIGURE 2: ^{13}C nmr spectra of calf skin gelatin: (a) and (b) observed for gelatin dissolved in 0.2 M KCl and adjusted to pH 7.0 (10-μsec pulse, 1-sec recycle time, 16,384 transients); (c) and (d) computer simulated.

TABLE I: ^{13}C Chemical Shifts (ppm) in Calf Skin Gelatin.[a]

Calf Skin Gelatin	Tentative Assignment	Residue Abundance[b]	Chemical-Shift Correlation with: Ribonuclease A[c]	Peptide	Amino Acid
12.8	Glu C^{δ}	72.1	15.5	11.7[e]	11.5[d]
15.9–16.3	Gln C^{δ}				15.0 or 18.6[f]
	Asp C^{γ}	47.3	16.5		15.2[d]
	Asn C^{γ}				17.8 or 19.0[f]
18.0–24.9	Carbonyls		15–23	16.1–25.2[c]	14.7–25.2[d]
57.0, 63.9, 64.6, 66.1	Phe $C^{\varphi i}$	12.6			
36.3	Arg C^{ζ}	47.9	36.0		36.1[d]
123.3	Hyp C^{γ}	94.1		122.7[h]	
126.1	Thr C^{β}	16.6		125.8[c]	126.5[d]
131.8	Ser C^{β}	39.2		131.6[e]	132.3,[d] 132.4[e]
132.5	Pro C^{α}	129.0		131.9,[c] 134.5[g]	132.0[d]
133.7	Ile C^{α}	11.3			132.7[d]
	Thr C^{α}	16.6		133.7[c]	132.1[d]
	Val C^{α}	19.5		134.1[e]	132.0,[d] 132.1[e]
134.3	Hyp C^{α}	94.1		135.5[h]	
	Ser C^{α}	39.2		137.3[e]	126.2,[d] 136.1[e]
	Tyr C^{α}	4.9		137.4[c]	
137.8	Phe C^{α}	12.6		137.6[c]	
	Hyp C^{δ}	94.1		137.6[h]	
	Gln C^{α}				138.2[f]
	Met C^{α}	3.9			138.4[d]
	Arg C^{α}	47.9			138.5[d]
139.5	Glu C^{α}	72.1		138.9[e]	137.9,[d] 137.8[e]
	Lys C^{α}	24.5		138.9[c]	138.3, 138.3[d]
	Leu C^{α}	24.0		140.0[e]	138.9,[d] 138.9[e]
	His C^{α}	4.8		140.2[c]	139.2[d]
141.6	Asn C^{α}				140.9[f]
	Asp C^{α}	47.3			140.4[d]
143.3	Ala C^{α}	106.0	142.8		142.0[d]
146.1	Pro C^{δ}	129.0		145.0[g]	147.5[d]
150.5	Gly C^{α}	336.5	150.0	150.3[e]	151.1,[d] 151.2[e]
151.1	Unknown				
152.4	Arg C^{α}	47.9	152.6		152.1[d]
153.6	Leu C^{β}	24.0	153.4	153.1[e]	152.6,[d] 152.8[e]
	Lys C^{ϵ}	24.5	153.4	153.4[c]	153.4,[c] 153.6[d]
	Phe C^{β}	12.6		156.0[c]	
	Asp C^{β}	47.3			156.0[d]
156.1	Ile C^{β}	11.3			156.5[d]
	Tyr C^{β}	3.6			156.8[d]
	Hyp C^{β}	94.1		157.0[h]	
	Asn C^{β}				157.6[f]
159.6	Glu C^{γ}	72.1		159.1[e]	159.1,[f] 159.1[c]
	Gln C^{γ}				161.6[f]
162.9	Met C^{β}	3.9			162.6[d]
	Val C^{β}	19.5		162.6[e]	163.4,[d] 163.6[e]
	Lys C^{β}	24.5		162.7[c]	162.7[c]
163.8	Met C^{γ}	3.9			163.5[d]
	Pro C^{β}	129.0		163.6,[c] 164.8[g]	163.9[d]
165.1	Glu C^{β}	72.1		164.8[e]	165.5,[d] 165.7[e]
	Arg C^{β}	47.9			165.1[d]
	Gln C^{β}				166.2[f]
166.8	His C^{β}	4.8		166.6[c]	166.2[c]
	Lys C^{δ}	24.5		166.6[c]	166.6[c]
	Ile C^{γ_1}	11.3			167.9[d]
168.7	Leu C^{γ}	24.0		168.5[e]	168.2,[d] 168.4[e]
	Pro C^{γ}	129.0		168.5,[c] 168.2[g]	169.2[d]
	Arg C^{γ}	47.9		168.5[e]	168.7[d]

TABLE I: (*Continued*)

Calf Skin Gelatin	Tentative Assignment	Residue Abundance[b]	Chemical-Shift Correlation with: Ribonuclease A[c]	Peptide	Amino Acid
171.2	Leu C^{δ_1}	24.0		170.6[e]	170.4,[d] 170.6[e]
	Lys C^{γ}	24.5		170.9[c]	171.2[d]
172.3	Leu C^{δ_2}	24.0	172.0	171.7[e]	171.5,[d] 171.6[e]
174.3	Thr C^{γ}	16.6		174.1[c]	173.1[d]
174.7	Val C^{γ_1}	19.5		175.0[e]	174.5,[d] 174.7[e]
175.5	Val C^{γ_2}	19.5		176.0[e]	175.7,[d] 176.0[e]
176.5	Ala C^{β}	106.6	175.6		176.3[d]
177.5	Ile C^{γ_2}	11.3	177.9		177.7[d]
178.5	Met C^{ϵ}	3.9	178.4		178.5[d]
182.5	Ile C^{δ_1}	11.3	182.5		181.3[d]

[a] At pH 7.0, parts per million upfield from CS_2; carbon types are given in accordance with IUPAC–IUB biochemical nomenclature. [b] Veis (1964). [c] Glushko *et al.* (1972). [d] Horsley and Sternlicht (1968); Horsley *et al.* (1970). [e] Gurd *et al.* (1971). [f] Allerhand *et al.* (1970). [g] Dorman *et al.* (1973). [h] This work. [i] Aromatic carbon atoms.

hydrogen bonds of lengths between 2.50 and 2.56 Å, all of the hydroxyl and carbonyl groups taking part. In addition, recent 220-MHz ^{1}H nmr data of poly(hydroxy-L-proline) (Torchia, 1972) also indicated that the heterocyclic ring possessed a single nonplanar conformation in aqueous solution. Therefore, the absence of disordered structure in solution for this macromolecule can be understood. On the other hand, it would appear that the tendency of poly(L-proline) to become disordered in solution is a consequence of its more open structure, lacking in hydrophobic stabilization, and the inability of the imino linkages to provide stabilization by hydrogen bonding.

From the integrated intensities of the proton-coupled and proton-decoupled spectra of poly(hydroxy-L-proline), the nuclear Overhauser enhancement (NOE) factors were calculated. They are 2.1, 2.0, 1.7, 2.1, and 1.7 for C=O, C^{α}, C^{β}, C^{γ}, and C^{δ} atoms, respectively.

^{13}C Nmr Spectrum of Calf Skin Gelatin. Figures 2a and 2b are the low- and high-field parts of a ^{13}C nmr spectrum of calf skin gelatin in 0.2 M KCl at pH 7.0. The observed chemical shifts are listed in column 1 of Table I. The corresponding resonances for ribonuclease A, oliogopeptides, and poly-α-amino acids, and simple amino acids are tabulated in columns 4, 5, and 6, respectively.

Line-by-line analysis of Figures 2a and 2b was guided by comparison with previously reported chemical shifts of ^{13}C nuclei in proteins (Gibbons *et al.*, 1970; Chien and Brandts, 1971; Allerhand *et al.*, 1970; Glushko *et al.*, 1972). Whenever the necessary data are not available, the comparison was made with oligopeptides and poly-α-amino acids (Gurd *et al.*, 1971; Christl and Roberts, 1972; Horsley and Sternlicht, 1968; Dorman *et al.*, 1973).

Those resonances which are assigned with reasonable certainty in Table I will not be elaborated further. Remarks need only to be made about those absorptions for which definite attributions cannot yet be made at this time. In the low-field region there are two resonances at 15.9 and 16.3 ppm; the candidates are Asp C^{γ}, Asn C^{γ}, and Gln C^{δ}. No plausible assignments can be made for the two well-resolved peaks at 24.2 and 24.9 ppm.

The region of the spectrum from 131.4 to 143.3 ppm consisted of five bands and is composed principally of C^{α} resonances. The sharp peak at 132.5 ppm probably arises from a single carbon nucleus which correlates well with the chemical shift of the Pro C^{α} nucleus. The shoulder at 131.4 ppm corresponds to the C^{β} of the seryl residues. Except for the C^{δ} atoms of Hyp, which are found to have a chemical shift of 137.6 ppm, the majority of the C^{α} resonances may be grouped into three ranges centered at approximately 133.7, 137.8, and 139.5 ppm, the assignments for which are suggested in Table I. The resonances at 141.4 and 143.3 ppm are best attributed to the C^{α} atoms in aspartic acid and alanyl residues, respectively.

The absorptions at high field are mostly accounted for in Table I.

Effect of pH. Two common effects of pH on the nmr spectrum expected are conformational sensitivity and degree of protonation. The former is of no consequence here because gelatin has a random-coil configuration and pH changes are not known to stimulate helix formation. The effect of protonation is, however, clearly seen in the spectra recorded from pH 2 to 10 at unit increments. At lower pH values, appreciable sharpening of individual resonances occurs, while higher pH values (>10) yield generally broadened resonance lines. Those resonances which show pronounced shifts with pH are given in Table II.

The observed shifts of C^{γ} and C^{δ} atoms of Glu with pH parallel the changes reported by Gurd *et al.* (1971). Significant effects might also be expected to accompany the deprotonation of the carboxylic side chain of aspartate, but the lack of model systems makes it impossible to be conclusive about this point. Finally, titration effects have been reported in the C^{δ} resonance of the lysyl residue when the pH was increased from 9.5 to 12.6 (Christl and Roberts, 1972). The effects on gelatin up to pH 10 are less pronounced; however, spectral changes near 166.3 and 171.2 ppm might originate from the titration effects on the C^{δ} and C^{γ} atoms of the lysyl residues, respectively.

Effect of Lyotropic Salts. Lyotropic salts are known to affect association–disassociation of collagen superstructures in the solid state (Chien and Chang, 1973) and helix–coil transitions of collagen–gelatin (von Hippel and Wong, 1962, 1963a,b; von Hippel and Schleich, 1969). In order to determine if these salts influence the molecular configuration of gelatin at ambient temperature, ^{13}C nmr spectra of gelatin in 2.0 M

TABLE II: ^{13}C Nmr Shifts (ppm) of C^{γ} and C^{δ} Resonances of Glutamic Acid Residues of Calf Skin Gelatin in 0.2 M Potassium Chloride.

Carbon Nucleus	pH of Measurement								
	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
C^{γ}	163.0	162.7	161.6	159.6	159.6	159.6	159.6	159.6	159.6
C^{δ}	16.0	15.5	15.0	13.0	13.0	12.8	12.5	12.3	12.3

KCNS and $CaCl_2$ as well as 0.5 M $(NH_4)_2SO_4$ were observed. The salts were chosen based on their known effectiveness in this regard.

The ^{13}C nmr spectra of gelatin in these salt solutions are not noticeably different from the "normal" spectrum. At the ambient temperature of 37°, used in these measurements, no significant conformational transition is expected (von Hippel and Schleich, 1969). Experiments are underway at lower temperatures and also to investigate the effect of lyotropic salts on the ^{13}C nmr spectra of collagen.

Spin–Lattice Relaxation Times. The partially relaxed Fourier transform method was used to determine T_1 values. Table III lists a collection of T_1 values in milliseconds for many ^{13}C nuclei. Also, shown in the table are T_1 values for native and denatured ribonuclease A.

Interpretations of spin–lattice relaxation times are usually based on Solomon's equation (1955). This equation takes on different forms depending upon the relaxation mechanism. There is compelling evidence that the overall relaxation of protonated carbon atoms in large molecules is overwhelmingly dominated by ^{13}C–H dipole–dipole interactions (Allerhand *et al.*, 1971; Glushko *et al.*, 1972; Schaefer, 1972). For this mechanism and if the molecular reorientation is isotropic which can be described by a single rotational correlation time (τ_R), then T_1 is given by

$$1/T_1 = (\hbar^2 n\gamma_C^2\gamma_H^2/10)[(1/12)J_0(\omega_C - \omega_H) + (3/2)J_1(\omega_C) + (3/4)J_2(\omega_C + \omega_H)] \quad (1)$$

where n is the number of directly bonded hydrogen atoms and γ_C and γ_H are the ^{13}C and ^1H gyromagnetic ratios, respectively. The spectral density functions are

$$J_0(\omega) = (24/15r^6)[\tau_R/(1 + \omega^2\tau_C^2)]$$
$$J_1(\omega) = (4/15r^6)[\tau_R/(1 + \omega^2\tau_R^2)] \quad (2)$$
$$J_2(\omega) = (16/15r^6)[\tau_R/(1 + \omega^2\tau_R^2)]$$

In eq 2, ω is the resonance frequency, r is the C–H distance, and τ_R is the correlation time for rotational correlation.

Equation 1, however, must be modified for atoms on side chains because they have additional freedom of rotation (Woessner, 1962; Wallach, 1967; Doddrell *et al.*, 1972). An effective correlation time, τ_{eff}, can be defined to be

$$(\tau_{eff})^{-1} = (\tau_R)^{-1} + (\tau_G)^{-1} \quad (3)$$

where τ_G is the internal rotational correlation time for segmental motion of backbone and rotation of side-chain functional groups. Doddrell *et al.* (1972) have presented some numerical results of T_1 for a tetrahedral methine carbon having a range of τ_G and τ_R.

Let us consider first the C^{α} resonances. In a native globular enzyme, the conformations of all backbone carbon atoms are determined by the tertiary structure of the molecule. There is little segmental motion and all the C^{α} nuclei should have the same τ_R which is the correlation time of the entire molecule. Consequently, they could have the same T_1 as well. This is found to be the case in ribonuclease A; T_1 is 35 msec for all C^{α} nuclei at pH 6.55 (Glushko *et al.*, 1972).

TABLE III: Spin–Lattice Relaxation Time (msec) of ^{13}C Nuclei in Gelatin.

Resonance	Gelatin[a]	RNase A[b]	Acid-Denatured RNase A[b]	Resonance	Gelatin	RNase A	Acid-Denatured RNase A
XC(=O)N	650			Arg C^{δ}	84		
Arg C^{ζ}	1400			Lys C^{ϵ}	280	278	295
Hyp C^{γ}	84			Glu C^{γ}	72		
Thr C^{β}		38	59	Lys C^{β}	150	66	93
Pro C^{α}	84	35	61	Pro C^{β}	80		
(133.7)[c]	84			(165.1)	84		
				Lys C^{δ}		188	151
(139.5)	92	35	35	Pro C^{γ}	80		
Ala C^{α}	120	35	67	Lys C^{γ}	115	100	121
Pro C^{δ}	92			Ala C^{β}	120	118	221
Gly C^{α}	55						

[a] This work. [b] Glushko *et al.* (1972). [c] The numbers in parentheses are the chemical shifts in parts per million of resonances given in Table I.

When the same enzyme is unfolded, independent motion of backbone segments becomes possible, which contributes to T_1. T_1 should become longer and different for various C^α nuclei depending upon the magnitude of τ_G. T_1 ranges from 82 to 107 msec in oxidized ribonuclease A at pH 1.4 (Glushko *et al.*, 1972). The spread is 55–120 msec in gelatin. The Gly C^α has T_1 which is 1/1.5 to 1/2.2 as large as the values of T_1 for C^α of all the other residues. In eq 1, the value of n is 2 for Gly C^α and 1 for other residues. On this basis, the difference in T_1 should be twofold. This is partly offset by the shorter τ_G for the Gly C^α atom than the C^α atoms in other residues because of less steric hindrance.

When $\tau_G \ll \tau_R$, τ_{eff} approaches τ_G and T_1 becomes independent of the overall conformation of the molecule. This is clearly demonstrated by the value of T_1 for Lys C^ϵ nuclei which is the same for both gelatin and native and denatured RNase A (Table III).

The value of τ_R for calf skin gelatin has not yet been determined but is expected to be of the order of 10^{-8} sec (Yguerabide *et al.*, 1970). Let us use this value of τ_R to estimate the magnitude of τ_G. For Pro C^α, Ala C^β, Lys C^β, and Lys C^ϵ nuclei, the respective τ_G values in nanoseconds are 0.2, 0.13, 0.1, and 0.045.

Spectra Simulation and Nuclear Overhauser Enhancement. Figure 2c,d is the computer-simulated ^{13}C nmr spectrum of gelatin based on the chemical shifts and amino acid composition given in Table I. Each resonance is assumed to have the same NOE and equal Lorentzian width of 8–9 Hz. Figure 2c,d reproduces very well the line positions of Figure 2a,b. On the other hand the agreement in peak heights is much less satisfactory.

At least two factors contribute toward the disparity in resonance intensities. The assumption of equal resonance line width could give rise to an error up to 40%. This estimate was based on the report that the line widths of carbon atoms in *trans*-poly(isoprene) range from 35 to 50 Hz (Schaefer, 1972).

The most important factor contributing toward the discrepancy in resonance intensity is probably the unequal NOE for the various carbon atoms. According to the theory based on a carbon–proton dipole relaxation mechanism (Kuhlmann *et al.*, 1970; Doddrell *et al.*, 1972), NOE is 2.988 in the extreme narrowing limit and is 1.04 for the slow rotational reorientation case. Whereas NOE for individual carbon atom can be determined for simple polymer molecules such as poly(hydroxy-L-proline), it becomes a formidable task for biomacromolecules because proton-coupled spectra are too poorly resolved for this purpose. However, if the composition is known, then it is possible to estimate NOE by comparing the resonance intensities of the observed and the computer-simulated spectra. The values of NOE for carbon atoms in gelatin were obtained from Figure 2, normalized by equaling the NOE of C^β, C^γ, and C^δ resonances of Hyp in gelatin with those observed for poly(hydroxy-L-proline). It is assumed that the side-chain carbon atoms in these two random coil molecules have comparable τ_R and τ_G values. The results are compiled in Table IV. Some trends can be discerned from the table.

There is almost no NOE for carbon atoms in the Glu and Asp residues which could be due to significant increase of correlation time because of hydrogen bondings involving these residues. Among the C^α atoms, those of Gly have much larger NOE. The carbon atoms far removed from the back bone apparently have the largest NOE.

In conclusion, more detailed properties, such as chemical shift, T_1, and NOE, for individual atoms in biomacromolecules can be determined with ^{13}C nmr than is possible with pmr. ^{13}C nmr is certain to become increasingly important as a tool for biochemical research.

TABLE IV: Nuclear Overhauser Enhancement of Individual ^{13}C Atoms in Gelatin.

	C^α	C^β	C^γ	C^δ	C^ϵ	C^ζ C(aromatic)
Thr		2.1	2.7			
Val			2.7			
Hyp		1.7	2.1	1.7		
Pro	1.4	2.1		1.6		
Ser		2.1				
Phe						2.1
Glu			1.0			
Arg				2.7	2.5	
Leu				2.1		
Asp	1.0					
Ala	1.2	1.5				
Gly	1.8					

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Carbon Monoxide Binding by Simple Heme Proteins under Photodissociating Conditions†

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ABSTRACT: This paper reports the binding of CO in the presence of photodissociating light by several simple heme proteins: sperm-whale myoglobin, *Aplysia* myoglobin, horseradish peroxidase, *Chironomus* hemoglobin, isolated α and β chains of human hemoglobin A, and *Gastrophilus* hemoglobin. In the presence of light, the CO binding curve maintains the shape of the simple (noncooperative) titration observed in the "dark." The apparent dissociation constant, given by $c_{1/2}$, shifts as a linear function of light intensity. Unfiltered light from the 150-W xenon arc increases the value of $c_{1/2}$ by about 1000-fold for sperm-whale myoglobin. The kinetics of approach to the steady state when the light is turned on and the return to equilibrium when it is turned off conforms to a simple one-step process. In relaxation experiments involving small perturbations of the steady state, the relaxation is linear in the sum of the concentrations of the reactants ($[\overline{Fe}]$ and $[\overline{CO}]$) for any given light intensity, which agrees with the idea that the CO combination rate constant (l') is unaffected by light. The value of $1\tau/\text{at}$ $([\overline{Fe}] + [\overline{CO}]) = 0$ is a linear function of light intensity, as expected on simple grounds. Steady-state and kinetic measurements permit the calculation of the quantum yield for CO photodissociation of the various hemoproteins (relative to sperm-whale myoglobin). Such calculations show that the quantum yield is temperature independent. Studies of the effect of temperature on the steady state and kinetics of the process are fully consistent with the hypothesis that photodissociation acts in addition to, and independently of, the dissociation process which occurs in the dark (l). Thus, the effective dissociation rate constant in the presence of light is given by $l + \omega I$, where ωI denotes the photochemical dissociation.

The photosensitivity of the carbon monoxide derivative of hemoglobin was originally reported by Haldane and Lorrain-Smith in 1895. The observations were extended to other hemoproteins by Warburg and collaborators (1949), who were the first to put the phenomenon on a quantitative basis. Since then it has been the subject of experiments by a variety of authors (Bucher and Kaspers, 1947; Gibson, 1956; Ainsworth and Gibson, 1957; Noble *et al.*, 1967; Brunori *et al.*, 1972). In such studies, values of the quantum yield were obtained by one of three types of measurements: (i) determination of the amount of ligand liberated by a pulse of light of known intensity; (ii) comparison of the relaxation time for the transition from equilibrium to the steady state produced by light with the relaxation time for the reverse process, either in the presence of one ligand (*e.g.*, CO) or of two ligands with different quantum yields (*e.g.*, CO and O_2); (iii) observations of the displacement of the ligand binding curve under the influence of a steady light.

The present study represents an extension of previously reported experiments (Brunori *et al.*, 1972) to the case of several "simple," one-site hemoproteins, namely: sperm-whale myoglobin, *Aplysia* myoglobin, horseradish peroxidase, *Chironomus* hemoglobin, isolated α and β chains of human hemoglobin A, and *Gastrophilus* hemoglobin. In these experiments we have made use of methods ii and iii, and have included an investigation of the effect of temperature.

The action of light is interpreted as affecting only the dissociation velocity constant in accordance with the simple scheme

$$\mathrm{Fe} + \mathrm{CO} \underset{l+\omega I}{\overset{l'}{\rightleftharpoons}} \mathrm{FeCO} \qquad (1)$$

where l' and l are the combination and dissociation velocity constants operative in the dark, I is the light intensity, and ω is a proportionality constant. According to this, the pseudo-equilibrium constant, L, for the steady state produced by light

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