A Magnetic Resonance Study of the Segmental Motion and Local Conformations of Poly(L-glutamic acid) in Aqueous Solutions

Akihiro Tsutsumi,[†] Bruno Perly, Alain Forchioni, and Claude Chachaty*

Service de Chimie Physique, C.E.N. de Saclay, B.P. No. 2, 91190 Gif-sur-Yvette, France. *Received February 3, 1978*

ABSTRACT: The segmental motions and the local conformations of poly(L-glutamic acid) in aqueous solutions have been investigated by magnetic resonance spectroscopy as a function of pH or pD and temperature. The ESR of spin-labeled PLGA with a nitroxide group attached to the end of the main chain or of a side chain indicates a sharp decrease of the mobility of this group at the random coil → α-helix transition. The analysis of the 250 MHz ^{1}H NMR spectra at different temperatures and pD shows the prevalence of the gauche and trans rotamers of the methylene groups of the side chains about the C_α–C_β and C_β–C_γ bonds, respectively. Deuteron line width measurements on the α- and γ-deuterated PLGA indicate a comparatively high segmental mobility with $10^{-10} < \tau_{\text{eff}} < 5 \times 10^{-10}$ s at room temperature for both the random coil and α-helix forms of the polymer. The nature of segmental motions of PLGA in neutral solution has been investigated by ^{13}C and ^{1}H T_1 measurements in magnetic fields of 23.5 and 58.7 kG. The relaxation data have been interpreted in terms of a quasi-isotropic reorientation of CH_α groups in the macromolecular chain assuming a distribution of correlation times τ_R with $\Delta H_R^\ddagger = 6.4$ kcal mol^{-1}. The model assessed for the reorientation of the β-methylene group is 120° jumps between three nonequivalent sites with $\Delta H_G^\ddagger = 6.4$ kcal mol^{-1}. The relaxation of $^{13}C_\gamma$ showing a large reorientational freedom of the γ-methylene group seems consistent with an overall isotropic motion with $\Delta H_T^\ddagger = 5.4$ kcal mol^{-1}. The influence of pD on the average dimensions of PLGA, investigated by the proton relaxation enhanced by a terminal nitroxide group, indicates in particular a marked shrinking of the macromolecular chain at the onset of the random coil → α-helix transition (pD 5.5).

I. Introduction

An important application of high resolution NMR in the field of macromolecules is the study of synthetic poly(amino acids) which may be considered as models for the conformation and the dynamical behavior of natural polypeptides.[1] During the past decade the variations of ^{1}H NMR chemical shifts associated with the helix–coil transition of polypeptides[2-7] were investigated in correlation with other physico-chemical methods,[1] in particular, optical spectroscopy (UV, IR, and circular dichroism), viscosimetry, and potentiometric titration. More recently considerable progress has been achieved in the knowledge of the conformations of polypeptides in solution by use of the high field ^{1}H NMR[6-8] and of the Fourier transform NMR spectroscopy which allows an accurate determination of the spin–lattice relaxation time T_1 in high resolution conditions.[9-11] In parallel to nuclear relaxation, the electron spin resonance of nitroxide spin labels appears as a useful method in the study of segmental motions of polypeptides.[12,13]

In the present work on poly(L-glutamic acid) (PLGA) in aqueous solution, the conformation of monomer residues has been determined by ^{1}H NMR spectroscopy, as a function of pD (or pH) and temperature. The incidence of the helix–coil transition on the segmental mobility of PLGA has been investigated by deuteron line-width measurements and by the ESR of the spin-labeled polymer at the end of the macromolecular chain and of the side chains. This latter technique, although providing less detailed information than the nuclear resonance and relaxation, is particularly convenient for rapid exploration in a wide range of experimental conditions, of the influence of the temperature, pH, and the concentration of the polymer on the segmental mobility of a macromolecule. We have completed this work by a more thorough study of the dynamical behavior of PLGA by ^{1}H and ^{13}C relaxation measurements, restricting ourselves to the neutral pD which corresponds to the random coil conformation of this polymer. These rather lengthy experiments are indeed precluded at low pD values (pD < 4.5) because of the precipitation and degradation of PLGA.

II. Experimental Section

(1) Magnetic Resonance Experiments. The ^{1}H and ^{13}C experiments were performed on a Cameca TSN 250 spectrometer ($\nu_H = 250$ MHz, $\nu_{^{13}C} = 63$ MHz) and a Varian XL100 spectrometer ($\nu_H = 100$ MHz, $\nu_{^{13}C} = 25.2$ MHz). The spin–lattice relaxation times T_1 of ^{1}H and ^{13}C were obtained by the inversion recovery method with a delay of five times the estimated value of T_1 between the 180°, τ, 90° sequences. The deuteron T_2 relaxation time was determined by line width measurements ($T_2^{-1} = \pi\Delta\nu_{1/2}$) at 15.3 MHz using the XL100 gyrocode observe accessory.

All the NMR experiments were performed on D_2O solutions of PLGA except for the determination of deuteron line widths. Instead of taking pD = pH + 0.4, it was assumed that pD was equal to the indications of the pH meter. That seems justified by the fact that the chemical shift and line width variations associated with the helix–random coil transition occur at the same measured pH values in H_2O and D_2O (see, for example, Figure 2).

The ESR experiments were performed at 9.2 GHz with a Varian E9 spectrometer.

(2) Materials. Poly(L-glutamic acid). L-glutamic acid γ-benzyl ester was prepared by the method of Blout[14] in 30% yield. It was then converted to the corresponding *N*-carboxyanhydride (NCA) by treatment with phosgene in tetrahydrofuran according to Goodman.[15]

The NCA was polymerized in DMF solution with *n*-hexylamine as initiator.[16] The anhydride to initiator ratio was 100. After 24 h at room temperature the poly(γ-benzyl L-glutamate) was precipitated in dry methanol. Viscosity measurement in DMF[17] indicated a molecular weight of 25 000 corresponding to a polymerization degree (DP) of 120.

Benzyl groups were then removed by HBr treatment in benzene according to the procedure of Idelson.[18] Poly(L-glutamic acid) thus obtained had a polymerization degree of 100 as shown by viscosity measurement in H_2O using the calibration of Rao.[19] Ultraviolet data at 260 nm indicated a complete removal of benzyl groups.

α-Deuterated Poly(L-glutamic acid). The L-glutamic acid **2d** (monomer) was prepared by a method[20] using controlled

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[†] Department of Polymer Science, Faculty of Sciences, Hokkaido University, Sapporo 060, Japan.

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racemization in deuterated medium followed by enzymatic resolution of the racemic derivative. The extent of deuteration in the α position was 96%. Specific rotation was identical to an original sample of L-glutamic acid. This confirms the optical purity of α-deuterated L-glutamic acid.

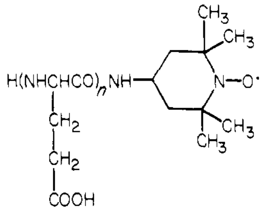
L-Glutamic acid **2d** was converted to the corresponding polymer in the same conditions as described above. Viscosity measurement indicated a polymerization degree of 110. α position was deuterated to the same extent as the corresponding monomer yielding further evidence that no racemization occurred during the entire process.

γ-Deuterated Poly(L-glutamic acid). The experimental procedure described in ref 21 was modified as follows: L-glutamic acid (6 g) (previously recrystallized from boiling D_2O) is suspended in 40 mL of 6 N DCl (99.5% D) in a glass ampule. After degassing and sealing, the mixture was warmed up to 70 °C causing dissolution. The homogeneous mixture was heated to 105–108 °C for 5 days. DCl in excess was removed by successive evaporation to dryness in the presence of water and the dry residue was dissolved in 20 mL of distilled water. Adjustment of pH to 3 with pyridine and addition of 20 mL of ethanol caused precipitation of L-glutamic-*4,4,*-d_2 acid. It was allowed to crystallize overnight at 4 °C, filtered, washed with ethanol, and dried in vacuo. The yield was 5.8 g (96%) of pure L-glutamic-*4,4*-d_2 acid with the same specific rotation as the original sample of untreated L-glutamic acid, indicating that no racemization occurred.

The NMR spectrum showed that 94–96% of the γ protons have been deuterated in these conditions.

The polymer was prepared from L-glutamic-*4,4*-d_2 acid in exactly the same conditions as the protio derivative. Molecular weight determination indicated a DP of 95.

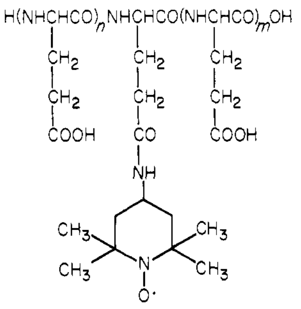
End-Labeled Poly(L-glutamic acid). γ-Methyl L-glutamic *N*-carboxy anhydride, prepared by phosgene treatment of γ-methyl L-glutamic acid, was polymerized in methylene dichloride with tempo-amine as an initiator. The anhydride to initiator ratio was 200. After 24 h, the polymer was isolated by precipitation in dry ethanol and dried in vacuo.



Removal of methyl groups was performed by saponification of the polymer in dioxane with ethanolic sodium hydroxide at room temperature. The precipitated spin labeled polymer was then dialyzed against distilled water for 10 days and freeze dried.

Molecular weight determination in water at pH 7 using the calibration of Rao[19] indicated a value of 10 000 corresponding to a DP of 67.

Side-Chain Labeled Poly(L-glutamic acid). Reaction between the free carboxyl group of PLGA and the amino group of the tempo-amine was performed using a water soluble carbodiimide as coupling reagent (CMC = 1-cyclohexyl-3-(2-morpholinoethyl)metho-*p*-toluene sulfonate); 450 mg (3×10^{-3} equiv COOH) of PLGA (DP100) was adjusted to pH 5 in 50 mL of water. After addition of 15 mg (3×10^{-5} mol L^{-1}) of CMC and 10 mg (5×10^{-3} mol) of tempo-amine, the pH was adjusted to 4.75. The final mixture was then incubated at 30 °C for 6 h.



Acidification to pH 2 with concentrated HCl led to precipitation of the polymer. It was then washed with 0.5 N acetic acid and 10^{-3} M HCl and dissolved in sodium bicarbonate solution. After dialysis for 5 days against distilled water and freeze drying, 500 mg of spin-labeled poly(L-glutamic acid)sodium salt are recovered.

III. Results and Discussion

(1) ESR of Spin-Labeled PLGA. In the whole range of our experimental conditions the separation of the three ESR lines of a nitroxide group attached to PLGA is constant and equal to the nitrogen isotropic hyperfine coupling $A_{\text{iso}}^{\text{N}}$. The reorientation correlation time of this group is obtained from the electron spin T_2 relaxation time measured from the peak-to-peak separation $\Delta\nu_{\text{ms}}$ of the first derivative of $m_{\text{N}} = 0, \pm 1$ lines of the ESR spectrum.[22,23]

$$T_2(m_{\text{N}})^{-1} = \pi 3^{1/2}\Delta\nu_{\text{ms}}(m_{\text{N}}) = \left[\frac{3b^2}{20} + \frac{4}{45}(\Delta\gamma B_0)^2 + \frac{b_2}{8}m_{\text{N}}^2 - \frac{4}{15}b\Delta\gamma B_0 m_{\text{N}}\right]\tau_{\text{c}} + X \tag{1}$$

with: $b = (4\pi/3)[A_{zz}^{\text{N}} - {}^1/_2(A_{xx}^{\text{N}} + A_{yy}^{\text{N}})] = 2\pi(A_{zz}^{\text{N}} - a_{\text{iso}}^{\text{N}})$ and $\Delta\gamma = -(\beta/\hbar)[g_{zz} - {}^1/_2(g_{xx} + g_{yy})] = (3\beta/2\hbar)(g_{zz} - g_{\text{iso}})$. B_0 is the value of the applied magnetic field for $g = 2.0023$, and X the contribution to T_2^{-1}, independent of τ_{c}. A_{xx}^{N}, A_{yy}^{N}, A_{zz}^{N}, and g_{xx}, g_{yy}, g_{zz} are the principal components of the hyperfine and g tensors, respectively. $A_{\text{iso}}^{\text{N}} = 47.77$ MHz and $g_{\text{iso}} = 2.00586$ have been obtained from the splitting and average positions of the three lines of the end-labeled and side-chain-labeled PLGA in aqueous solution at room temperature. $A_{zz}^{\text{N}} = 103.2$ MHz and $g_{zz} = 2.00233$ were given by half the separation and by the medium position of the two outermost peaks of the ESR spectra of PLGA at 100 K. The amplitude of the first derivative of an ESR absorption line being inversely proportional to the square of the linewidth $\Delta\nu_{\text{ms}}$, the correlation time τ_{c} may be obtained from the coefficient of m_{N} in eq 1

$$\tau_{\text{c}} = \frac{15T_2^{-1}(0)}{8b\Delta\gamma B_0}\left[\left(\frac{I_0}{I_+}\right)^{1/2} - \left(\frac{I_0}{I_-}\right)^{1/2}\right] \tag{2}$$

or from the coefficient of m_{N}^2

$$\tau_{\text{c}}^* = \frac{4T_2^{-1}(0)}{b^2}\left[\left(\frac{I_0}{I_+}\right)^{1/2} + \left(\frac{I_0}{I_-}\right)^{1/2} - 2\right] \tag{3}$$

where I_0, I_+, I_- are the respective amplitudes of the $m_{\text{N}} = 0, +1, -1$ lines.

The calculations have been done with $B_0 = 3240$ G, $b = 3.482 \times 10^8$ rad s^{-1}, and $\Delta\gamma = 4.65 \times 10^4$ rad s^{-1}.

The pH dependences of the reorientation correlation times obtained from eq 2 and 3 are qualitatively similar for the end-labeled and the side-chain-labeled PLGA: for both polymers τ_{c} increases upon lowering the pH and reaches plateau values above pH 7 and below pH 4, i.e., in the domains of the random coil and α helix forms of the polymer, respectively (Figure 1), the transition midpoint being located at pH 5 between 278 and 308 K. The reorientational freedom is lesser for the side-chain spin label than for the end-chain one, the relevant correlation times differing by ca. 50% at room temperature. All τ_{c} vs. pH curves show a minimum between pH 5 and 6, i.e., at the onset of the random coil → α helix transition, suggesting the existence of an intermediate state of the polymer which will receive further evidence in this work.

In the present case where the direction cosines between the principal axes of the diffusion tensor and those of the hyperfine and g tensors are unknown, the $\tau_{\text{c}}^*/\tau_{\text{c}}$ ratio may

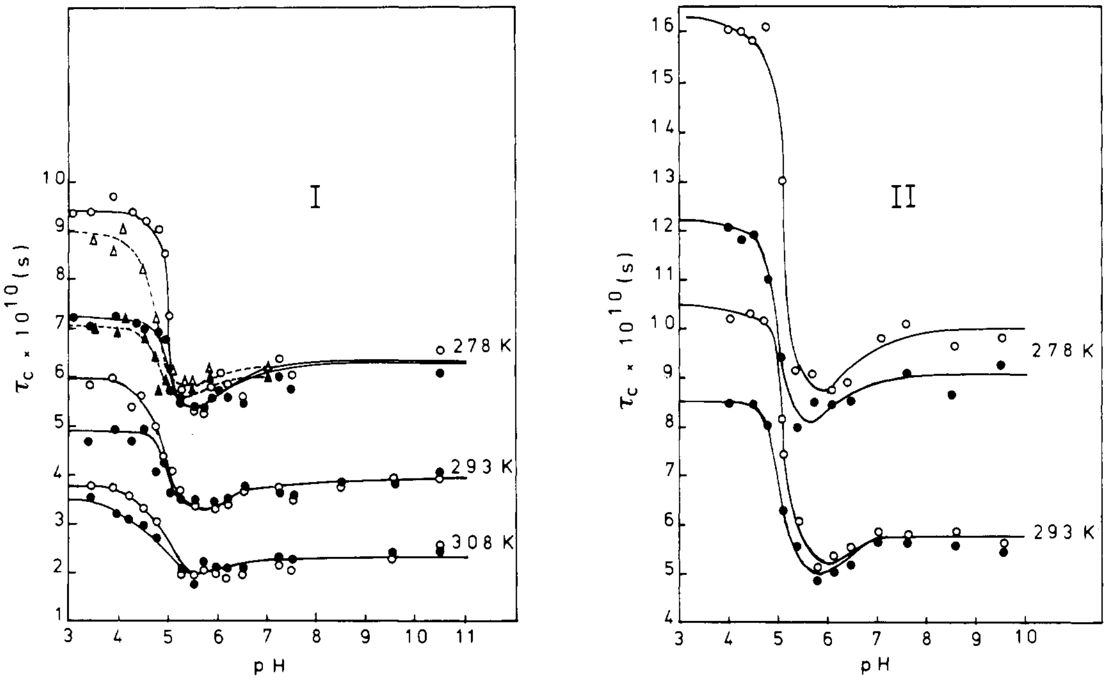


Figure 1. pH dependence of the reorientation correlation time of the nitroxide spin-label in the end-labeled (I) and side-chain labeled (II) PLGA, calculated from eq 2 (●, ▲) and 3 (○, △). ▲, △, and dotted lines: in the presence of 1 M NaCl.

be merely considered as a criterion for the anisotropy of the overall reorientation of the nitroxide group.[24]

For the end-labeled PLGA $\tau_c^*/\tau_c \approx 1$ down to pH 5 in the whole temperature range of our experiments, showing that the motion of the nitroxide spin label is quasiisotropic in the random coil state of the polymer. Below the random coil → α helix transition, the anisotropy of the motion of the spin label is evidenced by an increase of τ_c^*/τ_c which becomes more marked as the temperature is lowered (Figure 1).

Similar effects are observed in the case of the side-chain-labeled PLGA with larger values of τ_c^*/τ_c below pH 5. For the latter polymer a slight but significant difference between τ_c^* and τ_c is even observed at 278 K above pH 6.

The anisotropy of the motion of the nitroxide group is clearly not related to the overall motion of the macromolecule. In most of our experimental conditions τ_c^* and τ_c are indeed found in a range of 10^{-10} to 10^{-9} s, significantly shorter than the reorientation correlation time of the macromolecule expected to be of the order of 10^{-8}–10^{-7} s as shown later. The overall motion of the nitroxide group is the resultant of several anisotropic motions about C–C and C–N bonds. The orientations of the principal axes of the relevant diffusion tensor are likely to be randomly distributed in the coil state of the polymer where a large reorientational freedom exists. On the other hand, in the α helix form where the multiple segmental motions become more restricted, it is expected that the anisotropic character of the reorientation of the spin label at the extremity of the macromolecular chain or of a side chain becomes more apparent. Above pH 7, in the random coil state of the polymer where $\tau_c^*/\tau_c \simeq 1$ (isotropic motion) the temperature dependence of ESR line width yields $\Delta H^{\ddagger}$ = 5.8 kcal $\mathrm{mol^{-1}}$, $\Delta S^{\ddagger}$ = 4.3 cal $\mathrm{K^{-1}\,mol^{-1}}$ and $\Delta H^{\ddagger}$ = 5.4 kcal $\mathrm{mol^{-1}}$, $\Delta S^{\ddagger}$ = 2.3 cal $\mathrm{K^{-1}\,mol^{-1}}$ for the reorientation of the nitroxide group in the end-labeled and the side-chain-labeled PLGA, respectively. Moreover, this motion is nearly independent of the polymer concentration, at least up to 1 M in monomer units.

Under our experimental conditions, the ionic strength of the solution, estimated in $\mathrm{Na^+}$ concentration, was 0.05–0.1 M. In all magnetic resonance experiments reported here, the random coil → α helix transition midpoint is found between pH 5.0 and 5.1 independently of the sense of pH variations and of the temperature between 280 and 310 K. This is in agreement with results obtained under similar conditions by other techniques, e.g.: ^{13}C chemical shifts measurements,[25] potentiometric titrations,[26] and ultrasonic relaxation experiments.[27] The effect of the ionic strength on the helix → coil transition has been investigated by ESR line width measurements with [NaCl] = 1, 2, 3 M. The end- and side-chain-labeled PLGA behave similarly. No dramatic changes are observed in the limiting value of τ_c above pH 7. For [NaCl] = 1 M, the transition midpoint shifts to pH 4.5 (Figure 1), in disagreement with the ^{35}Cl line width measurement of Bryant[28] who finds pH 5 in the same conditions. At higher salt concentrations a further shift is observed toward lower pH values and precipitation of the polymer occurs before the completion of the transition.

Table I
Proton Vicinal Couplings and Chemical Shifts from 2,2′-Dimethyl-2-silapental 5-Sulfonate Obtained by Simulation of the 250-MHz NMR Spectrum of PLGA in Neutral D_2O at 298 K

	H_α	H_{β_1}	H_{β_2}	H_{γ_1}	H_{γ_2}
δ, ppm	4.320	2.044	1.932	2.300	2.248

	$\alpha\beta_1$	$\alpha\beta_2$	$\beta_1\beta_2$	$\beta_1\gamma_1 = \beta_2\gamma_2$	$\beta_1\gamma_2 = \beta_2\gamma_1$	$\gamma_1\gamma_2$
J, Hz	5.5	9	−15	8	6	−16

(2) Proton Chemical Shifts and Vicinal Coupling Constants. We have determined the influence of pD on the chemical shift of α, β, and γ protons of the PLGA of DP 100, 0.1 M in D_2O and of the α protons of this polymer, 0.5 M in H_2O. As reported previously[2] a large chemical shift variation is associated with the helix–coil transition at pH 5 (Figure 2), the steepest change being observed for the α proton. The doubling of the resonance of this proton, which has been discussed in a recent paper,[29] has not been observed in the case of the polymer under study.

The conformation of the side chains of PLGA has been obtained from the proton vicinal couplings (Figures 3 and 4; Table I). For the nondeuterated polymer, these cou-

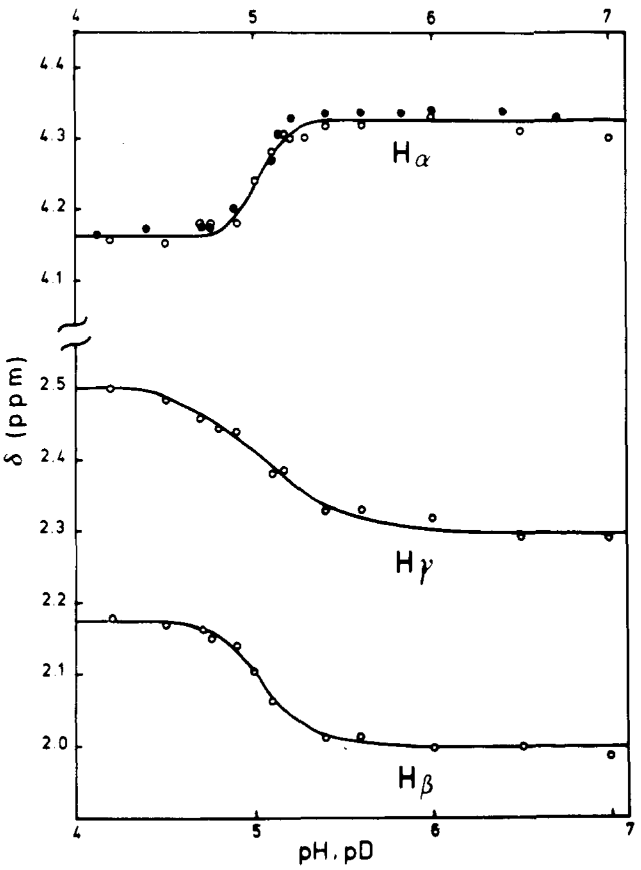


Figure 2. pD or pH dependence of the chemical shifts of H_α, H_β, and H_γ in ppm from DSS (2,2-dimethyl-2-silapentane 5-sulfonate) at 294 K: ○, PLGA 0.1 M in D_2O; ●, PLGA 0.5 M in H_2O.

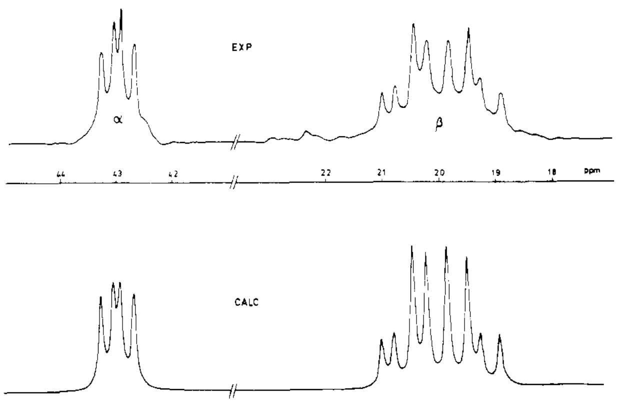


Figure 3. NMR spectrum (250 MHz) of α-deuterated PLGA in neutral D_2O at 294 K. The calculated spectrum has been obtained with parameters given in Table I. The chemical shifts are in ppm from DSS. The broadening of H_β resonances is due to unresolved couplings with γ deuterons.

plings are too poorly resolved to be measured with a sufficient accuracy below pD 6. Upon deuteration in the γ position, the resolution of the H_α and H_β resonances is appreciably improved and the $J_{\alpha\beta}$ coupling remains resolved down to pD = 4 in spite of the line broadening.

The proton vicinal couplings have been interpreted in terms of the torsion angles χ_1 and χ_2 about the C_α–C_β and C_β–C_γ bonds by use of the Karplus relation:

$$J_{HH} = A \cos^2 \chi + B \cos \chi \tag{4}$$

with $A = 12.47$ Hz and $B = -1.03$ Hz,[30] the trans ($\chi = 180°$) and gauche ($\chi = \pm 60°$) coupling constants being $J_t = 13.5$ Hz and $J_g = 2.6$ Hz. For the CH_α–$CH_{\beta1\beta2}$ residue the probabilities of the T, G, and G′ rotamers of the carbon backbone (Figure 5) are given by:

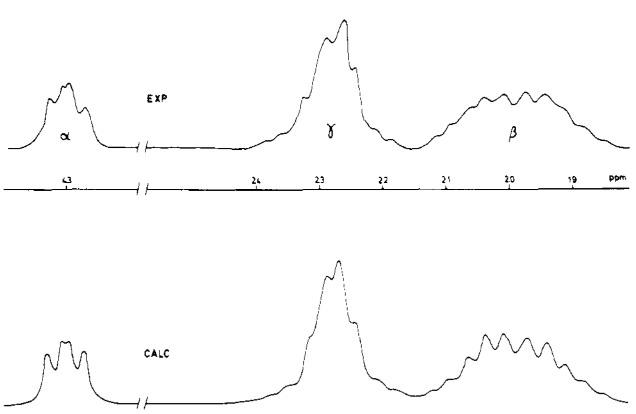


Figure 4. NMR spectrum (250 MHz) of normal PLGA in neutral D_2O at 294 K. Same conditions as Figure 3.

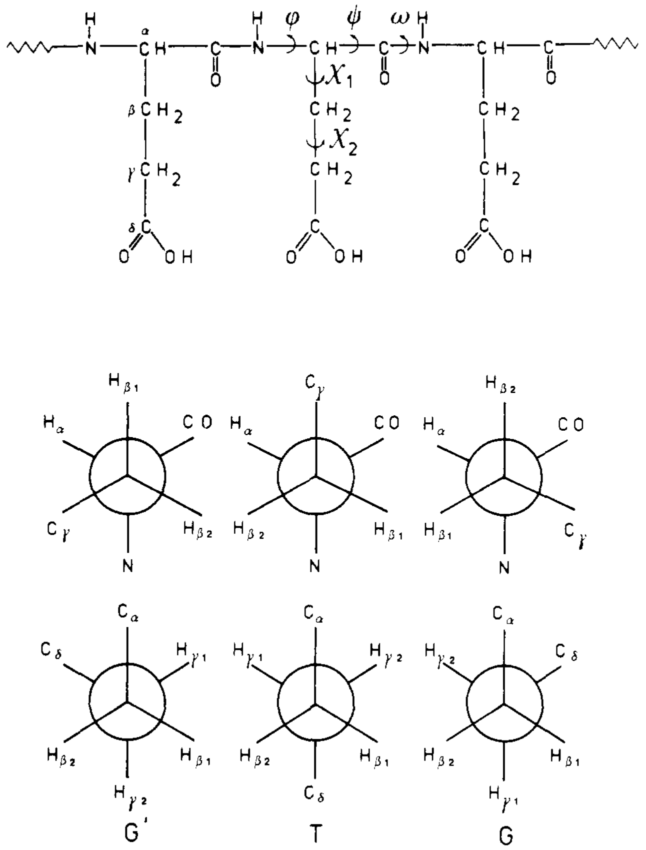


Figure 5. Definition of torsion angles of PLGA (see ref 6) and of the different rotamers of β- and γ-methylene groups.

$$P_T = \frac{J_{\alpha\beta1} - J_g}{J_t - J_g}$$

$$P_{G'} = \frac{J_{\alpha\beta2} - J_g}{J_t - J_g} \tag{5}$$

$$P_G = 1 - P_T - P_{G'}$$

and we obtain $P_T \simeq 0.25$, $P_{G'} = 0.60$, and $P_G = 0.15$ at room temperature for $5 < \mathrm{pD} < 7$. While the probabilities of the three rotamers are not very temperature dependent at pD ∼ 7, $P_{G'}$ and P_T increase upon warming at pD = 4.9 (Figure 6).

The probabilities of the T, G, and G′ rotamers about the C_β–C_γ bond (Figure 5) are obtained likewise from the $J_{\beta\gamma}$

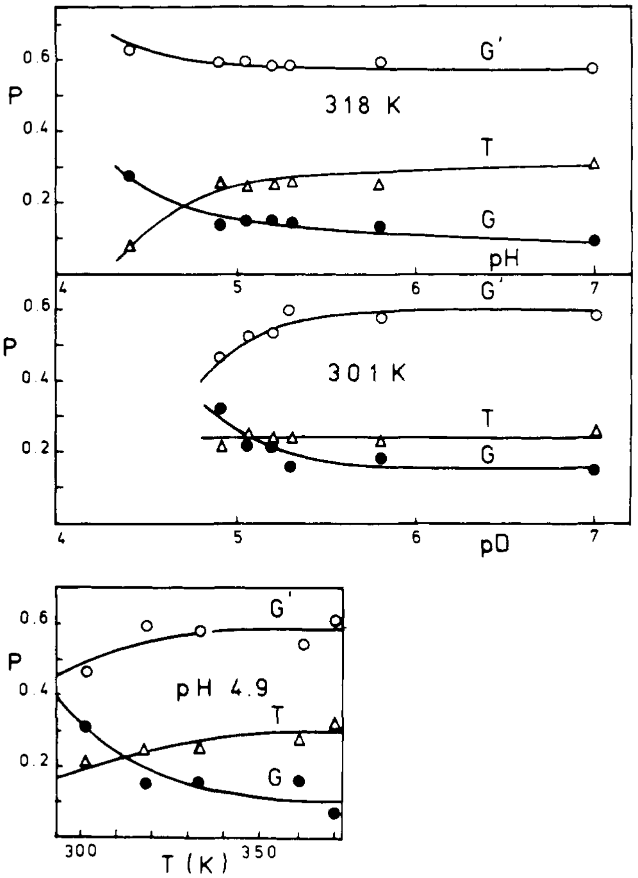


Figure 6. Temperature and pD dependences of the probabilities of the different rotamers of the β methylene groups derived from the $H_{\alpha}H_{\beta}$ vicinal couplings.

couplings with the values of J_g and J_t given above by equations

$$P_T = \frac{J_{\beta1\gamma1} - J_g}{J_t - J_g} = \frac{J_{\beta2\gamma2} - J_g}{J_t - J_g} \tag{6a}$$

$$P_G = \frac{J_{\beta1\gamma2} - J_g}{J_t - J_g} \tag{6b}$$

yielding $P_T = 0.5$, $P_G = 0.31$, and $P_{G'} = 0.19$, or by equations

$$P_T = \frac{J_{\beta1\gamma1} - J_g}{J_t - J_g} = \frac{J_{\beta2\gamma2} - J_g}{J_t - J_g} \tag{7a}$$

$$P_G = \frac{J_{\beta2\gamma1} - J_t - P_T}{J_g - J_t} \tag{7b}$$

which give $P_T = 0.5$, $P_G = 0.19$, $P_{G'} = 0.31$, or by equations

$$P_G = \frac{J_{\beta1\gamma2} - J_g}{J_t - J_g} \tag{8a}$$

$$P_T = \frac{J_{\beta1\gamma1} - J_t - P_G}{J_g - J_t} \tag{8b}$$

which give $P_T = 0.38$, $P_G = P_{G'} = 0.31$.

It appears therefore that the trans rotamer about C_{β}–C_{γ} is predominant.

(3) Nuclear Spin Relaxation. (a) pH Dependence of Proton and Deuteron Relaxations. The pH or pD dependence of the segmental mobility of PLGA has been investigated by ^1H and ^2D relaxation. It has been found that the α, β, and γ proton spin–lattice relaxation times at 250 MHz are nearly independent on the polymer concentration up at least to 0.5 M in neutral solution in agreement with ESR experiments on the spin-labeled polymers. Below pD = 5 the concentration of PLGA was taken at most equal to 0.1 M to avoid the line broadening due to the packing of macromolecules in the α helix form. Under these conditions, the ^{13}C experiments which need concentrations ≥0.5 M to obtain a sufficient sensitivity are not convenient to study segmental motions.

The proton T_1 measurements have been performed at 25 °C as a function of pD on a 0.1 M solution of PLGA deuterated in γ position. While the T_1 of β protons which are mainly relaxed by their mutual dipolar interaction does not change with pD (Figure 7), the relaxation time of H_{α} decreases below pD = 5 because of the slowing down of segmental motions and/or of a change in the local conformation of monomer residues. This point has been examined by deuteron line width measurements, in the same conditions, on the PLGA deuterated in α and γ positions (Figure 8). The relaxation of a deuteron bound to a carbon is indeed essentially induced by its quadrupole moment and nearly independent of dipolar interactions with nearby protons. It yields the same information about motions as the ^{13}C dipolar relaxation induced by attached protons, allowing in the present case the performing of experiments at higher dilutions (0.1 M). The effective

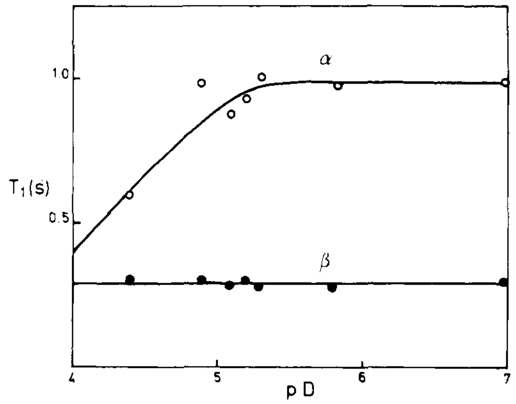


Figure 7. Spin–lattice relaxation times of H_{α} and H_{β} as a function of pD at 250 MHz for γ-deuterated PLGA 0.1 M in D_2O at 294 K.

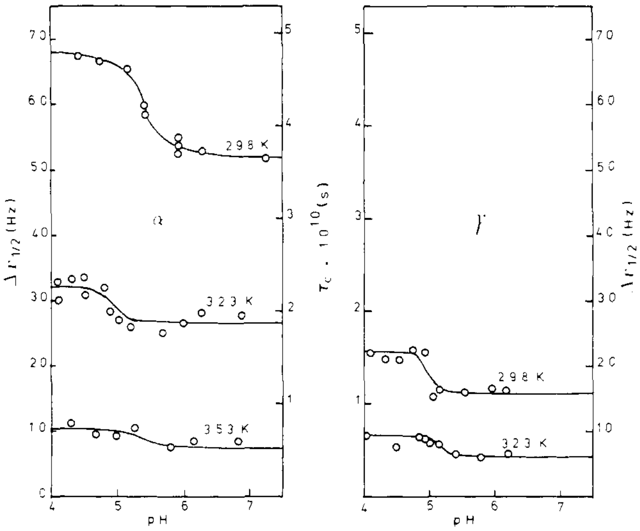


Figure 8. pD dependence of the effective reorientation correlation time of α-methine and γ-methylene groups from deuteron line width measurements.

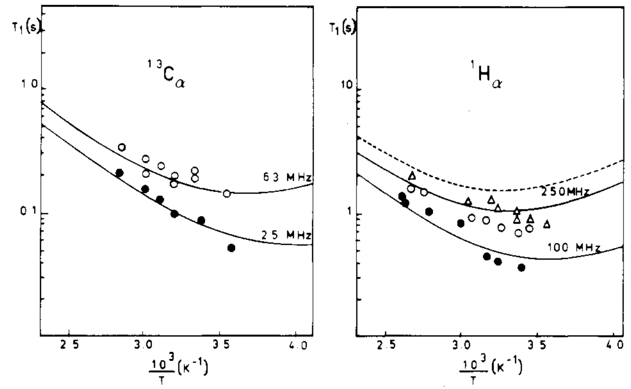


Figure 9. T_1 relaxation times of H_α and C_α in magnetic fields of 23.5 kG (●) and 58.7 kG (○ or △). The $T_1 = f(T^{-1})$ curves have been computed with $\Delta H_R^\ddagger = 6.4$ kcal mol^{-1}, $(\tau_R)_0 = 1.38 \times 10^{-14}$ s, and $\gamma = 0.67$ for the assumed isotropic segmental motion of the macromolecular chain. In the diagrams of H_α the dotted line and △ correspond to the γ-deuterated PLGA at 250 MHz. The proton $T_1 = f(T^{-1})$ curves are calculated for a jumping motion of $CH_{2\beta}$ taking into account long-range dipolar interactions with other surrounding protons (see the text). In ^{13}C and ^1H experiments the PLGA concentration is 0.5 and 0.1 M, respectively, and pD $\simeq$ 7.

correlation time τ_c is related to the quadrupolar transverse relaxation time T_{2q} by the equation

$$T_{2q}^{-1} = \pi\Delta\nu_{1/2} = \frac{3}{80}\left(\frac{e^2qQ}{\hbar}\right)^2\left[3\tau_c + \frac{5\tau_c}{1 + \omega_D^2\tau_c^2} + \frac{2\tau_c}{1 + 4\omega_D^2\tau_c^2}\right] \quad (9)$$

where $e^2qQ/\hbar = 1.1 \times 10^6$ rad s^{-1} is the quadrupolar coupling constant of the deuterium bound to a carbon and $\omega_D = 9.68 \times 10^7$ rad s^{-1}, the deuteron Larmor frequency in a field of 23.5 kG.

At room temperature, the random coil → α helix transition brings about a 30% increase of the α deuteron line width, the limiting values of the effective τ_c being 3.7 $\times 10^{-10}$ and 4.8 $\times 10^{-10}$ s in the coil and helix states, respectively. As the temperature is raised, the broadening observed at the transition diminishes and disappears completely above 353 K (Figure 8). The T_1 variations observed for the α proton at room temperature are therefore related, at least in part, to local conformational changes evidenced by vicinal coupling measurements. A similar behavior is observed for γ deuterons (Figure 8) at room temperature and above showing a restriction of the reorientational freedom of the side chains of PLGA in the helix form as evidenced also by ESR experiments on the side-chain-labeled polymer.

(b) Proton and Carbon-13 Relaxation in Neutral Solutions. The segmental motion of PLGA at neutral pD has been investigated by measurement of ^{13}C and ^1H spin–lattice relaxation times at two frequencies.

The temperature dependence of the T_1 of $^{13}C_\alpha$ at 25 and 63 MHz (Figure 9) has been calculated by assuming that the segments of the macromolecular chain in the random-coil state undergo an isotropic motion. The ratio of the relaxation times at these two frequencies suggests the existence of a distribution of correlation times τ_c about a mean value $\bar{\tau}_R$. We have adopted in our calculation the Cole–Cole distribution:

$$L(s) = \frac{1}{2\pi}\,\frac{\sin(\gamma\pi)}{\cosh(\gamma s) + \cos(\gamma\pi)} \quad (10)$$

with $s = \ln(\tau_c/\bar{\tau}_R)$, $0 \leq \gamma \leq 1$ being the parameter giving the width of the distribution. $\gamma = 1$ corresponds to a single correlation time.

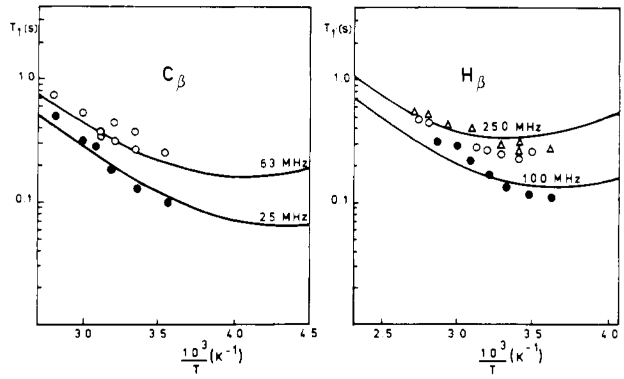


Figure 10. Temperature dependence of the C_β and H_β spin–lattice relaxation times. Same experimental conditions as Figure 9. Normal PLGA: ●, $H_0 = 23.5$ kG; ○, $H_0 = 58.7$ kG. γ-Deuterated PLGA: △, $H_0 = 58.7$ kG. The $T_1 = f(T^{-1})$ curves have been computed for a jumping motion of $CH_{2\beta}$ coupled with the quasiisotropic motion of CH_α, taking $\Delta H_G^\ddagger = \Delta H_R^\ddagger = 6.4$ kcal mol^{-1} and $\gamma_G = \gamma_R = 0.67$ for the distribution of τ_G and τ_R. For β protons we take only into account the geminal dipolar coupling.

For an exponential autocorrelation function, the expression of the spectral density at the Larmor frequency ω becomes[31]

$$J(\omega) = \frac{1}{2\omega}\,\frac{\cos(1-\gamma)(\pi/2)}{\cosh(\gamma\ln(\omega\bar{\tau}_R)) + \sin(1-\gamma)(\pi/2)} \quad (11)$$

the ^{13}C relaxation rate being given by

$$T_1^{-1} = \frac{1}{10}\hbar^2\gamma_H^2\gamma_C^2 r_{CH}^{-6}[J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_C + \omega_H)] \quad (12)$$

we have taken $r_{CH} = 1.09$ Å.

Different enthalpies of activation $\Delta H^\ddagger$ and preexponential factors $(\tau_R)_0$ defined by $\bar{\tau}_R = (\tau_R)_0 \exp(\Delta H_R^\ddagger/RT)$ may be obtained from the quasilinear dependence of T_1 upon $1/T$ at 25 MHz according to the values assumed for γ and for the position of the T_1 minimum which cannot be determined experimentally. The most appropriate set of parameters has been selected by fitting simultaneously theoretical $T_1 = f(T^{-1})$ curves to the relaxation times obtained at 25 and 63 MHz. We have thus obtained for C_α, $\Delta H_R = 6.4$ kcal mol^{-1}, $(\tau_R)_0 = 1.38 \times 10^{-14}$ s ($T_{min} = 250$ K at 25 MHz), and $\gamma = 0.67$ (Figure 9).

To estimate whether the relaxation of C_α is influenced by the overall tumbling motion of the macromolecule, we have calculated the relevant correlation time by the Stokes–Einstein relation:

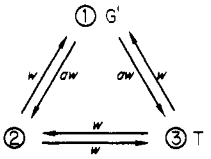
$$\tau_{overall} = \frac{4\pi\langle R_G^2\rangle^{3/2}\eta}{3kT} \quad (13)$$

where η is the viscosity of water and R_G the radius of gyration of the macromolecule given by:

$$\langle R_G^2\rangle = Cl^2 \times \mathrm{DP} \quad (14)$$

where C is a constant taken equal to 1.3 for a DP = 100 as estimated by Flory[32] in the case of poly(alanine) and l is the length of the monomer unit. We find thus $(\langle R_G\rangle^2)^{1/2} = 43$ Å and 1.9×10^{-8} s $< \tau_{overall} < 1.1 \times 10^{-7}$ s for the temperature varying from 280 to 370 K. The effective correlation time τ_R determined by ^{13}C relaxation or ESR being of the order of 10^{-10} to 10^{-9} s in the same temperature range, there is no appreciable contribution of the overall motion of PLGA.

The relaxation of the β carbon at 25 and 63 MHz (Figure 10) has been interpreted in terms of an anisotropic motion of the methylene group about the C_α–C_β bond, undergoing an isotropic reorientation, as assumed for the segments of the macromolecular chain. For such an anisotropic motion, Woessner[33] has proposed the models of a stochastic rotational diffusion or a jumping motion between three equivalent positions. The analysis of the α and β protons spin–spin coupling suggests to us however that a more appropriate model is the jumping between three nonequivalent sites corresponding to the T, G, and G′ conformers (Figure 6). For simplicity we shall assume that the G and T conformers have the same probability of 0.2. The jumping motion about the C_α–C_β bond may then be represented by the following scheme,



where w is the jumping rate between sites 2 and 3 corresponding to the G and T conformers, and a is a constant.

The time-dependent probabilities of the three sites are then:

$$P_1(t) = \frac{1}{2a+1} + \alpha \exp\left(-\frac{t}{\tau_{G1}}\right) \tag{15a}$$

$$P_2(t) = \frac{a}{2a+1} - \frac{1}{2}\alpha \exp\left(-\frac{t}{\tau_{G1}}\right) + \beta \exp\left(-\frac{t}{\tau_{G2}}\right) \tag{15b}$$

$$P_3(t) = \frac{a}{2a+1} - \frac{1}{2}\alpha \exp\left(-\frac{t}{\tau_{G1}}\right) - \beta \exp\left(-\frac{t}{\tau_{G2}}\right) \tag{15c}$$

where $\tau_{G1} = [w(2a + 1)]^{-1}$, $\tau_{G2} = (3w)^{-1}$, α and β being constants determined by the initial conditions. The equilibrium probabilities are $P_1(\infty) = 1/(2a + 1) \simeq 0.6$, $P_2(\infty) = P_3(\infty) = a/(2a + 1) \simeq 0.2$, and therefore $a \simeq {}^1/_3$.

The case of three equivalent sites treated by Woessner[33] corresponds to $a = 1$.

For the model of jumping motion between three nonequivalent sites, one obtains by use of eq 15 the spectral density:

$$J_h(\omega) = 2K_h[Rf(\tau_R) + Sf(\tau_1) + Tf(\tau_2)] \tag{16}$$

where $K_0 = {}^4/_5$, $K_1 = {}^2/_{15}$, and $K_2 = {}^8/_{15}$

$$f(\tau) = \frac{\tau}{1 + \omega^2\tau^2}$$

$$R = A + B\,\frac{a^2 - 1}{(2a + 1)^2}$$

$$S = \frac{9}{2}B\frac{a}{(2a + 1)^2}$$

$$T = \frac{3}{2}B\frac{a}{(2a + 1)^2}$$

$$A = {}^1\!/_4(3 \cos^2 \Delta - 1)$$

$$B = {}^3\!/_4(\sin^2 2\Delta + \sin^4 \Delta)$$

and $\tau_{1,2} = (\tau_R^{-1} + \tau_{G1,2}^{-1})^{-1}$, τ_R being the correlation time of the isotropic motion of the C_α–C_β axis and Δ the angle between C_β–$H_{\beta1,2}$ and C_α–C_β. The relaxation rate of $^{13}C_\beta$ under proton noise decoupling is then:

$$(T_1^{-1})_{C_\beta} = {}^1\!/_{10}\gamma_C^2\gamma_H^2\hbar^2 r_{CH}^{-6}[RF(\tau_R) + SF(\tau_1) + TF(\tau_2)] \tag{17}$$

with:

$$F(\tau) = J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_C + \omega_H)$$

In the absence of a distribution of correlation times $J(\omega) = \tau(1 + \omega^2\tau^2)^{-1}$. Assuming, however, that $\tau_{G1} = k_1\tau_R$ and $\tau_{G2} = k_2\tau_R$, the same distribution function holds for the three correlation times of eq 16 and $J(\omega)$ is given by eq 11. In the expressions of $F(\tau_1)$ and $F(\tau_2)$ the $\bar{\tau}_R$ of eq 12 is replaced by $\bar{\tau}_{1,2} = (\bar{\tau}_R^{-1} + \bar{\tau}_{G1,2}^{-1})^{-1}$.

A satisfactory agreement between the $^{13}C_\beta$ relaxation times at 25 and 63 MHz and the calculated $T_1 = f(T^{-1})$ calculated curves has been achieved by taking the values of $(\tau_R)_0$ and ΔH_R given above for the isotropic segmental motion of the macromolecular chain and $\Delta H_G = 6.4$ kcal mol^{-1} with $(\tau_{G1})_0 = 3.53 \times 10^{-15}$ s and $(\tau_{G2})_0 = 1.96 \times 10^{-15}$ s for the jumping motion of the methylene group about C_α–C_β (Figure 10). The same value of $\gamma = 0.67$ was taken for the width of the distribution of τ_R, τ_{G1}, and τ_{G2}.

The spin-lattice relaxation time of β protons in ordinary and in γ-deuterated PLGA has been measured as a function of the temperature. Nearly the same results are obtained for both polymers (Figure 10) and it may be assumed that their mutual dipolar interaction is the major relaxation process so that the same treatment as for the β carbon may be applied, the relaxation rate being given by:

$$T_1^{-1} = {}^3\!/_{10}\gamma_H^4\hbar^2 r_{HH}^{-6}[RG(\tau_R) + SG(\tau_1) + TG(\tau_2)] \tag{18a}$$

where:

$$G(\tau) = J(\omega_H) + J(2\omega_H) \tag{18b}$$

$J(\omega_H)$ and $J(2\omega_H)$ are given by eq 11.

In the calculation of the $T_1 = f(T^{-1})$ curves at 100 and 250 MHz, we have taken the same parameters as for C_β with $r_{HH} = 1.78$ Å and $\Delta = 90°$ (Figure 10).

The relaxation of the α proton is mainly induced by the β protons of the same monomer unit, the length of the H_α–H_β vectors changing with time, because of the rotation of the methylene group (Figure 11). A similar problem has been treated by Woessner for the equivalent three-states model.[34] In the present case where the three sites are not equivalent, the method of calculation summarized above for the relaxation of C_β and H_β has been modified to take into account the time dependence of the length and orientation of H_α–H_β. The relaxation rate of H_α induced by one of the β protons, within a short time interval following a 180° pulse, is given by:

$$T_1^{-1} = {}^3\!/_{20}\gamma_H^4\hbar^2[V_RG(\tau_R) + V_1G(\tau_1) + V_2G(\tau_2)] \tag{19}$$

The expressions of coefficients V_R, V_1, and V_2 as a function of the angle χ are given in the appendix. The most probable positions of $H_{\beta1}$ and $H_{\beta2}$ corresponding to the G′ rotamer are defined by $\chi_{1\beta1} = 180°$ and $\chi_{1\beta2} = 300°$.

The contribution of γ protons to the dipolar relaxation of H_α is more difficult to evaluate exactly since the length and orientation of H_α–H_γ vectors depend upon the two rotational angles χ_1 and χ_2. We have therefore replaced $H_{\gamma1}$ and $H_{\gamma2}$ by their projection on the $C_\alpha C_\beta$ axis, using the same procedure as for β protons, with $\chi_{1\gamma} = 300°$. Even taking into account this last contribution, it appears that the observed relaxation rate of H_α is nearly half the expected one (Figure 9). We have therefore estimated the dipolar interactions of this proton with α and β protons of the vicinal monomer residues. The effective interproton distances were obtained by use of hard core potentials, varying randomly the angles φ, ψ, and χ by 20° steps. These calculations yield $\langle r_{\alpha\alpha'}\rangle = 3.65$ Å and $\langle r_{\alpha\beta'}\rangle = 4.15$ Å. The contribution of γ protons of vicinal units to the

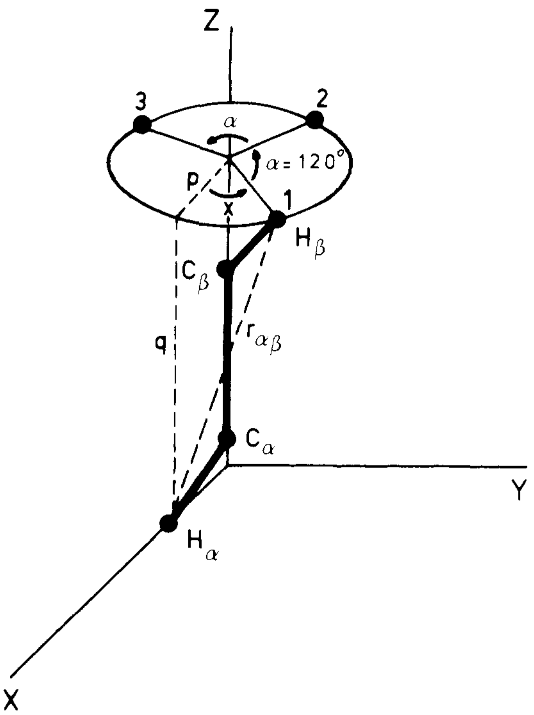


Figure 11. Definition of geometrical parameters involved in the calculation of dipolar interactions of H_α with H_β (eq 19, see the Appendix).

relaxation of H_α was found to be negligibly small as confirmed by comparison of the results obtained with the normal and γ deuterated PLGA. The additional relaxation rate of H_α due to the protons of vicinal units is then:

$$(T_{1\mathrm{ext}}{}^{-1})_\alpha = {}^3\!/_{10}\hbar^2\gamma_{\mathrm{H}}{}^4[2\langle r_{\alpha\alpha'}\rangle^{-6} + 4\langle r_{\alpha\beta'}\rangle^{-6}]G(\tau_{\mathrm{R}}) \qquad (20)$$

$G(\tau_{\mathrm{R}})$ being given by eq 11 and 18b for an isotropic motion and a distribution of correlation time with the same values of the above mentioned parameters.

The ^{13}C and ^{1}H relaxation in the γ methylene group, which possesses a large reorientational freedom, has been treated under the simplified assumption of an isotropic motion (Figure 12). A fairly good fit of the $^{13}C_\gamma$ relaxation times at 25 and 63 MHz is obtained with $\Delta H_{\mathrm{R}}{}^{\ddagger} = 5.4$ kcal mol^{-1}, $(\tau_{\mathrm{R}})_0 \simeq 10^{-14}$ s, and $\gamma = 0.9$ for the Cole–Cole distribution of τ_{R} (eq 10 and 11). Taking the same parameters for the relaxation of γ protons, with $r_{\mathrm{HH}} = 1.78$ Å, the calculated relaxation times are appreciably larger than the experimental ones, owing probably to the entanglement of the macromolecular chain which brings about dipolar interactions with protons of remote monomer residues.

(4) Proton Relaxation Enhancement in Spin-Labeled PLGA. The information provided by the different magnetic resonance experiments reported here concerns essentially the local motions of PLGA and the conformations of monomer residues. To determine at least qualitatively the effect of pH on the overall conformation of the macromolecule we have measured the relaxation of β and γ protons enhanced by a nitroxide group at one extremity of the polymer chain, which give an estimate of the average distance of monomer units to the chain end. The less reliable results of T_1 measurements on H_α which are perturbed by the nearby HDO resonance are not given here.

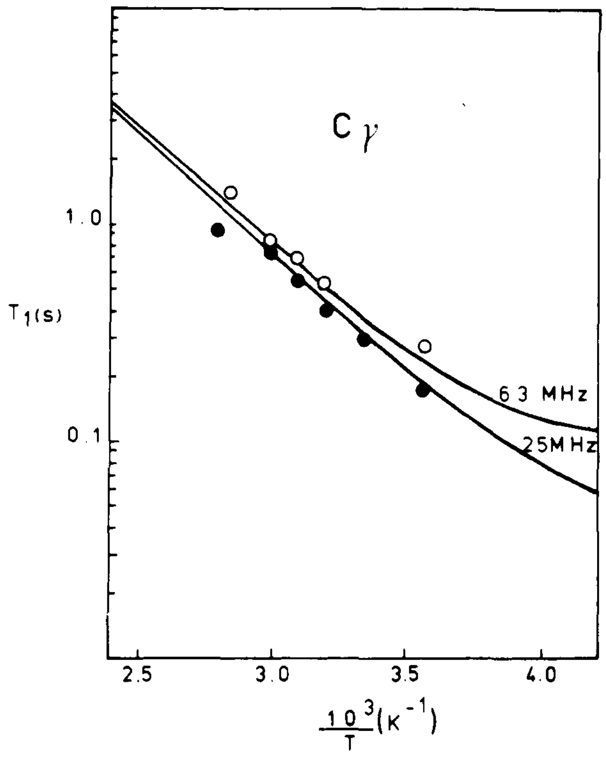


Figure 12. Temperature dependence of the C_γ spin–lattice relaxation times for $H_0 = 23.5$ kG (●) and $H_0 = 58.7$ kG (○). Same experimental conditions as Figures 10 and 11. Assumed isotropic motion of the γ-methylene group with $\Delta H_{\mathrm{R}}{}^{\ddagger} = 5.4$ kcal mol^{-1}, $(\tau_{\mathrm{R}})_0 = 10^{-14}$ s, and $\gamma = 0.9$.

The proton relaxation experiments on 0.1 M solutions of the end-chain-labeled PLGA of DP 70 have been done at 295 K at a spectrometer frequency of 250 MHz. The relaxation rates were corrected for the contribution of the proton–proton dipolar interactions measured in the same conditions on a nonlabeled sample of PLGA. We have also verified that the relaxation rates measured on the spin-labeled PLGA are not concentration dependent below 0.3 M in monomer units, showing that the intermolecular dipolar interactions are negligibly small under our experimental conditions. The proton relaxation rate induced by the paramagnetic probe is given by the Solomon equation[35] modified for the case where the $T_{1\mathrm{e}}$ and $T_{2\mathrm{e}}$ electron-spin-relaxation times are different:

$$T_{1\mathrm{P}}{}^{-1} = \frac{2}{15}\gamma_{\mathrm{H}}{}^2\gamma_{\mathrm{S}}{}^2\hbar_2 S(S+1)r_{\mathrm{HS}}{}^{-6}\left[\frac{3\tau_{\mathrm{c1}}}{1+\omega_{\mathrm{H}}{}^2\tau_{\mathrm{c1}}{}^2} + \frac{7\tau_{\mathrm{c2}}}{1+\omega_{\mathrm{S}}{}^2\tau_{\mathrm{c2}}{}^2}\right] \qquad (21)$$

where r_{HS} is the proton to nitroxide group distance, γ_{S} is the electron magnetogyric ratio, $S = {}^1/_2$ is the total electron spin, and $\omega_{\mathrm{S}} = 1.033 \times 10^{12}$ rad s^{-1} is the electron Larmor frequency for $H_0 = 58.7$ kG. The τ_{c1} and τ_{c2} correlation times are defined by:

$$\tau_{\mathrm{c1,2}} = (\tau_{\mathrm{c}}{}^{-1} + T_{1,2\mathrm{e}}{}^{-1})^{-1} \qquad (22)$$

where τ_{c} is the reorientation correlation time of the nitroxide spin label given by eq 2 or 3.

At room temperature τ_{c} is of the order of 5×10^{-10} s for the end-labeled PLGA and $T_{2\mathrm{e}}$ is found between 2×10^{-8} and 5×10^{-8} s in an applied magnetic field of 3 kG. $T_{2\mathrm{e}}$ is expected to be larger in a field of 58.7 kG. $T_{1\mathrm{e}}$ is always larger than $T_{2\mathrm{e}}$ and increases as $1 + \omega_{\mathrm{s}}{}^2\tau_{\mathrm{c}}{}^2$ with the electron Larmor frequency[36] so that $T_{1\mathrm{e}}{}^{-1}$ and $T_{2\mathrm{e}}{}^{-1}$ are negligible compared to $\tau_{\mathrm{c}}{}^{-1}$ and $\tau_{\mathrm{c1}} \approx \tau_{\mathrm{c2}} \approx \tau_{\mathrm{c}}$. Moreover, as $\omega_{\mathrm{s}}{}^2\tau_{\mathrm{c}}{}^2 >> 1$ for $B_0 = 58.7$ kG, eq 21 may be replaced by:

$$T_{1\mathrm{P}}{}^{-1} = \frac{3}{10}\gamma_{\mathrm{H}}{}^2\gamma_{\mathrm{S}}{}^2\hbar^2 r_{\mathrm{HS}}{}^{-6}\frac{\tau_{\mathrm{c}}}{1+\omega_{\mathrm{H}}{}^2\tau_{\mathrm{c}}{}^2} \qquad (23)$$

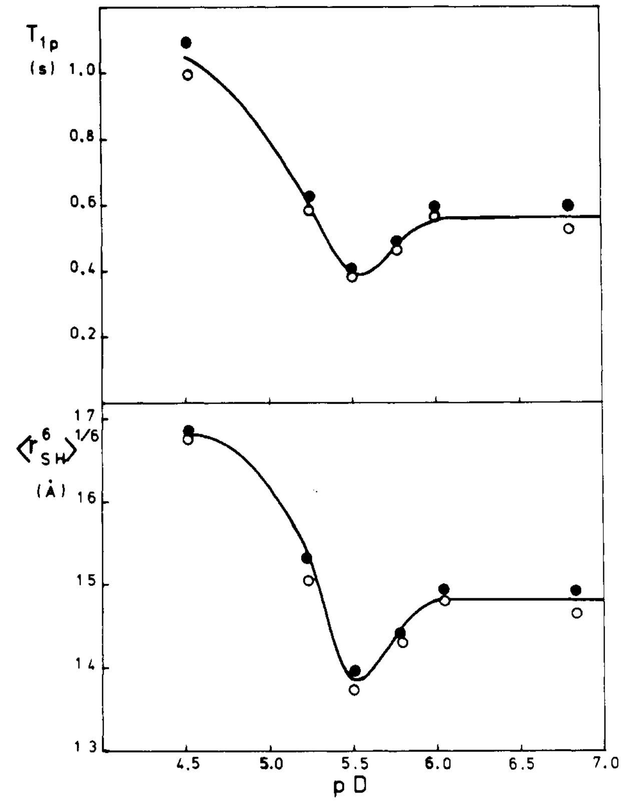


Figure 13. H_β and H_γ spin–lattice relaxation at 250 MHz (T = 295 K) enhanced by a nitroxide group at the macromolecular chain end and corresponding proton–spin label average distances.

The average distances $\langle r_{HS}{}^6\rangle^{1/6}$ of β and γ protons to the paramagnetic chain end of PLGA are obtained from their paramagnetic relaxation times and the correlation times τ_c given by eq 2 or 3 which yield nearly the same results in the present case. Figure 13 shows that $\langle r_{HS}{}^6\rangle^{1/6}$ is larger for the α helix form than for the random coil, passing through a minimum at pD = 5.5. This result may be explained by a conformational change of the macromolecule which passes from a rodlike form in the α helix state to a more compact globular form between pD = 5 and 6 and expands in the random-coil state because of Coulombic repulsion between charged carboxyl groups. This observation is consistent with the pH dependence of the viscosity of PLGA in dioxane–water solution[37] and of poly-L-lysine in aqueous solutions[38] which shows a minimum at the helix–coil transition.

IV. Conclusion

The electron spin resonances such as ^1H, ^2D, and ^{13}C relaxation experiments concur to show that the dynamical behavior of PLGA in aqueous solutions is governed by fast segmental motions with effective correlation times of the order of 5×10^{-10} s at room temperature, rather than by Brownian motions of the macromolecule; these results are consistent with the ^{35}Cl NMR line width study of Miller et al.[39] The α deuteron line width measurements, while indicating significant changes in the segmental mobility of the macromolecular backbone of PLGA at the helix-coil transition, show that the hindrance of segmental motions in the helix form is less stringent than in the case of poly(γ-benzyl L-glutamate)[9] and poly(L-lysine).[11] ^1H and ^{13}C relaxation experiments suggest cooperative motions of the β-methylene and α-methine groups of PLGA which have the same enthalpy of activation and the same correlation time distribution. The γ deuteron line width measurements and the ESR of the side-chain spin-labeled PLGA indicate on the other hand a marked pH dependence of the reorientational freedom of the terminal groups of side chains which may be assessed to an increase of the free volume available for these groups with the degree of ionization of carboxyl groups, which move apart under the influence of Coulombic interactions. Finally, the proton relaxation enhancement by a nitroxide group at one end of the macromolecular chain gives an insight on the changes in the average dimensions of PLGA at the helix–random coil transition and suggests the existence of an intermediate, more compact, state of this polymer at the onset of this transition as proposed by Wade et al.[10] for the denaturation of polypeptides.

V. Appendix.

The expression of relaxation rate of H_α induced by dipolar coupling with $H_{\beta 1}$ and $H_{\beta 2}$ (eq 19) has been established for the case of a jumping motion between sites 1, 2, and 3 (Figure 11), the sites 2 and 3 being equivalent. These three sites are defined by angles:

$$\chi_{11} = \chi, \qquad \chi_{12} = \chi + \alpha, \qquad \chi_{13} = \chi - \alpha$$

the relevant $H_\alpha H_\beta$ distances being given by:

$$r_{01}{}^2 = 2p^2(1 \cos \chi) + q^2$$

$$r_{02}{}^2 = 2p^2[1 - \cos (\chi - \alpha)] + q^2$$

$$r_{03}{}^2 = 2p^2[1 - \cos (\chi + \alpha)] + q^2$$

In the case where α = 120°, the coefficients of eq 19, $V_R = C_{A1} + C_{B1} + C_{C1}$, $V_1 = C_{A2} + C_{B2} + C_{C2}$, $V_2 = C_{A3} + C_{B3} + C_{C3}$, are:

$$C_{A1} = \frac{2}{(2a+1)^2}\Big[(p^2 - q^2 - p^2 \cos \chi)r_{01}{}^{-5} + a\Big\{p^2 - q^2 + p^2 \cos\Big(\chi - \frac{\alpha}{2}\Big)\Big\}r_{02}{}^{-5} + a\Big\{p^2 - q^2 + q^2 \cos\Big(\chi + \frac{\alpha}{2}\Big)\Big\}r_{03}{}^{-5}\Big]^2$$

$$C_{A2} = \frac{a}{(2a+1)^2}\Big[(p^2 - q^2 - p^2 \cos \chi)r_{01}{}^{-5} - \Big\{p^2 - q^2 + p^2 \cos\Big(\chi - \frac{\alpha}{2}\Big)\Big\}r_{02}{}^{-5} - \Big\{p^2 - q^2 + p^2 \cos\Big(\chi + \frac{\alpha}{2}\Big)\Big\}r_{03}{}^{-5}\Big]^2$$

$$C_{A3} = \frac{a}{(2a+1)}\Big[\Big\{p^2 - q^2 + p^2 \cos\Big(\chi - \frac{\alpha}{2}\Big)\Big\}r_{02}{}^{-5} - \Big\{p^2 - q^2 + p^2 \cos\Big(\chi + \frac{\alpha}{2}\Big)\Big\}r_{03}{}^{-5}\Big]^2$$

$$C_{B1} = \frac{12p^2q^2}{(2a+1)^2}\Big[(1 - \cos \chi)r_{01}{}^{-10} + a^2\Big\{1 + \cos\Big(\chi - \frac{\alpha}{2}\Big)\Big\}r_{02}{}^{-10} + a^2\Big\{1 + \cos\Big(\chi + \frac{\alpha}{2}\Big)\Big\}r_{03}{}^{-10} + a\Big\{\frac{1}{2} - \cos \chi + \cos\Big(\chi - \frac{\alpha}{2}\Big)\Big\}r_{01}{}^{-5}r_{02}{}^{-5} + a^2\Big(\frac{1}{2} + \cos \chi\Big)r_{02}{}^{-5}r_{03}{}^{-5} + a\Big\{\frac{1}{2} - \cos \chi + \cos\Big(\chi + \frac{\alpha}{2}\Big)\Big\}r_{01}{}^{-5}r_{03}{}^{-5}\Big]$$

$$C_{B2} = \frac{6p^2q^2a}{(2a+1)^2}\left[4(1-\cos\chi)r_{01}^{-10} + \left\{1 + \cos\left(\chi - \frac{\alpha}{2}\right)\right\}r_{02}^{-10} + \left\{1 + \cos\left(\chi + \frac{\alpha}{2}\right)\right\}r_{03}^{-10} - 2\left\{\frac{1}{2} - \cos\chi + \cos\left(\chi - \frac{\alpha}{2}\right)\right\}r_{01}^{-5}r_{02}^{-5} + \left(\frac{1}{2} + \cos\chi\right)r_{02}^{-5}r_{03}^{-5} - 2\left\{\frac{1}{2} - \cos\chi + \cos\left(\chi + \frac{\alpha}{2}\right)\right\}r_{01}^{-5}r_{03}^{-5}\right]$$

$$C_{B3} = \frac{6p^2q^2a}{(2a+1)}\left[\left\{1 + \cos\left(\chi - \frac{\alpha}{2}\right)\right\}r_{02}^{-10} + \left\{1 + \cos\left(\chi + \frac{\alpha}{2}\right)\right\}r_{03}^{-10} - \left(\frac{1}{2} + \cos\chi\right)r_{02}^{-5}r_{03}^{-5}\right]$$

$$C_{C1} = \frac{3}{2}\frac{p^4}{(2a+1)^2}\left[2\{3 - 4\cos\chi + \cos 2\chi\}r_{01}^{-10} + 2a^2\left\{3 + 4\cos\left(\chi - \frac{\alpha}{2}\right) + \cos 2\left(\chi - \frac{\alpha}{2}\right)\right\}r_{02}^{-10} + 2a^2\left\{3 + 4\cos\left(\chi + \frac{\alpha}{2}\right) + \cos 2\left(\chi + \frac{\alpha}{2}\right)\right\}r_{03}^{-10} - a\{3 + 4\cos(\chi - \alpha) - 2\cos 2\chi - 2\cos(2\chi - \alpha)\}r_{01}^{-5}r_{02}^{-5} - a^2(3 + 4\cos\chi + 2\cos 2\chi)r_{02}^{-5}r_{03}^{-5} - a\{3 + 4\cos(\chi + \alpha) - 2\cos 2\chi - 2\cos(2\chi + \alpha)\}r_{01}^{-5}r_{03}^{-5}\right]$$

$$C_{C2} = \frac{3}{2}a\frac{p^4}{(2a+1)^2}\left[4\{3 - 4\cos\chi + \cos 2\chi\}r_{01}^{-10} + \left\{3 + 4\cos\left(\chi - \frac{\alpha}{2}\right) + \cos(2\chi - \alpha)\right\}r_{02}^{-10} + \left\{3 + 4\cos\left(\chi + \frac{\alpha}{2}\right) + \cos(2\chi + \alpha)\right\}r_{03}^{-10} + \{3 + 4\cos(\chi - \alpha) - 2\cos 2\chi - 2\cos(2\chi - \alpha)\}r_{01}^{-5}r_{02}^{-5} - \left\{\frac{3}{2} + 2\cos\chi + \cos 2\chi\right\}r_{02}^{-5}r_{03}^{-5} + \{3 + 4\cos(\chi + \alpha) - 2\cos 2\chi - 2\cos(2\chi + \alpha)\}r_{01}^{-5}r_{03}^{-5}\right]$$

$$C_{C3} = \frac{3}{4}a\frac{p^4}{(2a+1)}\left[2\left\{3 + 4\cos\left(\chi - \frac{\alpha}{2}\right) + \cos(2\chi - \alpha)\right\}r_{02}^{-10} + 2\left\{3 + 4\cos\left(\chi + \frac{\alpha}{2}\right) + \cos(2\chi + \alpha)\right\}r_{03}^{-10} + (3 + 4\cos\chi + 2\cos 2\chi)r_{02}^{-5}r_{03}^{-5}\right]$$

The complete theoretical calculations of ^1H and ^{13}C relaxation rates for a jumping motion between three nonequivalent sites 40 (eq 16, 17, 18, and 19) will be published in the near future.

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