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Spectroscopic Evidence for Spatially Sequential Amide Bond Formation in Plant Homopolygalacturonans

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ABSTRACT: To investigate further the physicochemical properties of plant homopolygalacturonans (PGA), solutions and solid suspensions of this polymer were reacted with a carbodiimide reagent in the presence of a paramagnetic nucleophile. Upon reaction we discovered that when as few as 2.5% of the carboxyl functional groups had been labeled, the nitroxyamide EPR powder patterns were significantly broadened. This broadening effect was the same for reactions occurring in either solution or the solid state. Dimer-only interspin distances (d) were approximately 12 Å which is close to carboxyl group spacings within this polymer. Line-width- and relaxation-time-related parameters approached those of nitroxyl radicals randomly spaced in a lattice upon competitive reaction with a nonparamagnetic amine or by the partial reduction of the labeled lattice with ascorbate. These data all indicate that the reaction at some initial site creates a greater potential for nucleophilic attack by the paramagnetic amine with near-neighbor sites than other sites along the polyanion's main chain. This preference for near-neighbor sites is suggested to result in galacturonan polymers with sequential blocks of amides.

Introduction

Polygalacturonans (PGA) are important constituents of matrix polysaccharides in higher plant cortical tissues, certain algal cell walls, and extracellular polysaccharide coats of some prokaryotes.[1-3] In higher plants these polymers consist mainly of simple linear blocks of α-(1→-4)galactopyranuronic acid[2] which can have, in vivo, variable methyl ester contents.[4] The biological importance of PGA is related to its ability to modulate the mono- and divalent cation concentration within the plant cell wall network[5] and act as cell wall polymer adhesive agents.[4] The behavior of these biopolymers is not well understood[6,7] and is of interest since the interactions between charged polymers and their environment can dominate[8] higher order structure, hydration, H-bonding, and assemblage and, thus, affect the stability of the cell wall network.

Previous workers have shown that certain PGA-containing polymers have a cooperative divalent cation binding behavior in solution[9-15] and a sequential mechanism in the solid state.[6,7] Since this cooperativity could be related to the higher order structure and self-complexation of these polyanionic species, a more detailed knowledge of the chemical and physical properties of PGA is directly relevant to their spatial arrangement and function in cell walls[16] and other structural assemblies. In this report we provide evidence that PGA carboxyl functional groups undergo sequential "activation" and subsequent nucleophilic attack by 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4AT) in a fashion remarkably similar to divalent cation binding observed before by PGA-containing polymers[6,7] in plant cell walls.

Experimental Section

Poly(galacturonic acid) (H⁺ form, PGA) and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) were purchased from Sigma Chemical Company, St. Louis, MO. 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4AT) and the borane:tetrahydrofuran complex (BH₃:THF) were purchased from Aldrich Chemical Company, Milwaukee, WI.

Spin-Labeling Reaction Conditions.[17-22] For each spin-labeling reaction,[19] 0.1 g of PGA was dissolved in 40 mL of H_2O (solution reactions) or suspended in 50% (v/v) Me₂SO:H₂O (solid-state reactions) with concomitant vigorous agitation. After 4AT (1×10^{-6}–5×10^{-3} mol) was added, the pH was slowly adjusted to 4.75 with 0.1–1 N HCl whereupon 0.1 g of EDC was

added to the reaction mixture at 20–30-min intervals to a total of 1 g. The reaction mixture was kept at a constant pH at 22 °C by the continual addition of 0.1 N HCl with a Radiometer (reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned) pH stat. The reaction was considered complete only after the addition of HCl was no longer required to maintain a static pH (usually after 4–5 mL was added). The solution reaction mixtures were exhaustively dialyzed for 3 days. The solid PGA treatments were first spun down (10000g) and washed extensively with Me₂SO:H₂O (50% v/v) prior to dialysis; at the highest concentration of 4AT, the reacted polymer became soluble in Me₂SO:H₂O. All samples were protected from incidental light by wrapping vessels containing 4AT with aluminum foil. After lyophilization the samples were stored in vacuo over dehydrated silica gel at –10 °C. Representative PGA–amides of 4AT were tested, after several weeks of storage as above, for reduction of the label to the hydroxylamine form by adding 50 μL of 3mM K₃Fe(CN)₆ to 50 μL of dissolved PGA-amide in a capillary tube; no increase in EPR spectral intensity was observed over 1 h, indicating that very little of the spin label was reduced during the above reaction procedures or subsequent storage.

For competitive reactions the procedure was identical with the above except that in addition to 4AT (2×10^{-3} mol/treatment) aniline was added in a 30-, 10-, or 2-fold molar excess relative to the paramagnetic nucleophile (4AT). For control purposes, 1.12 g of the 4AT-amide of PGA (9.63 mol % attached label) was prepared as described above, split into seven equal fractions and partially reduced in solution with various levels of ascorbate (2×10^{-5}–1×10^{-4} mol). Samples were redialyzed and freeze dried as before. Table I provides NMR data on the chemical shifts and ¹H–¹³C scalar coupling constants of reduced 4AT and its corresponding amide of PGA (the hydroxylamine). These data show that no N-acylurea, a possible byproduct in the use of EDC,[18-20] was detectable; the chemical shifts for the free and covalently bound hydroxylamine were similar; the ¹H-induced ¹³C splitting patterns were those expected for these compounds; and the presence of a 170.5 ppm carbonyl peak was found in the labeled PGA sample, indicating that an amide was formed.

Hydrolysis of PGA-Anilides.[23] To increase solubility in organic solvents, anilides of PGA were derivatized to the propionate esters by slowly adding 1 g of the PGA-amide derivative to warm (50 °C) formamide in a reflux apparatus equipped with a mechanical stirrer. This mixture was refluxed for 1 h until a stiff paste was formed whereupon ca. 9 g of pyridine was added slowly dropwise. This mixture was allowed to cool to approximately 30 °C whereupon 9 g of propionic anhydride was added slowly and allowed to react 6 h. The resultant solution was cooled to ca. 20 °C, and 150 mL of prechilled 2% HCl, with 14 g of ice, was added. A precipitate was formed, isolated by centrifugation, and washed 3× with 0.5% HCl. The slurry was then freeze dried and dissolved in acetone; precipitation and washings with diethyl

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Table I
Relative Intensity, ^{13}C Chemical Shift (δ), and ^{1}H–^{13}C Scalar Coupling Constants (J) of Reduced 4AT and Its Amide of Polygalacturonic Acid (PGA-Amide)

| rel intensity | source | δ, ppm | | J, Hz |
		reduced 4AT[a]	reduced PGA-amide	
0.34[b]	quaternary (singlet)	60.1[c]	59.5	
0.15	tertiary (doublet)	44.1	42.0	143.43
0.36	secondary (triplet)	42.1	40.7	128.94
0.20	primary (quartet)	30.5	29.0	122.91
0.21	primary (quartet)	19.8	18.3	122.91
0.80	carbonyl, acid		175.0	
0.23	carbonyl, amide		170.5	
1.00	anomeric		99.2	
0.98	COH		78.0	
0.95	COH		72.0	
1.94	COH		70.3	

[a] Compounds were reacted with ascorbate,[40,41] passed through an anion-exchange column (the polymer was dialyzed and freeze dried) to remove unreacted ascorbate and its oxidation product, dihydroascorbate, and concentrated in vacuo on a rotary evaporator at 30 °C. See Figure 2 for the appropriate structure. [b] All PGA-amide signals were normalized to the anomeric carbon signal. Reduced 4AT signals were virtually identical with similar resonances of the reduced PGA-amide from the standpoint of relative intensity. The PGA-amide had ca. 19 mol % N–O$^{\bullet}$, by EPR double integration, prior to reduction. COH ≡ carbohydrate ring carbons. [c] [^{2}H]Me$_2$SO was used as an internal reference with ^{2}H$_2$O as the solvent.

ether followed thereafter. The vacuum-dried propionate esters of the PGA-anilides isolated in the last step were dispersed in 175 mL of THF under a dry N$_2$ atmosphere. In order to reduce the unreacted carboxyl group (C-6), 50 mL of BH$_3$:THF complex was added dropwise and the solution stirred overnight. Absolute ethanol was added dropwise slowly to quench the reaction. The reaction product (mixed polygalactose:polyanilide) was precipitated and washed with diethyl ether, dried under vacuum, suspended in pH 10 H$_2$O in order to cleave the remaining propionate esters, exhaustively dialyzed, and freeze dried. Mild hydrolysis of the galactose–galactose linkages in 0.1 N H$_2$SO$_4$ at 100 °C for 30 min was followed by neutralization with BaCO$_3$. The solution volumes were reduced, and BaSO$_4$ was removed by centrifugation; this process was repeated thrice. The resultant solutions were then dialyzed (1000 molecular weight cutoff tubing) and freeze dried. ^{13}C NMR spectra (400 MHz for ^{1}H) were obtained with a single pulse-gated ^{1}H decoupling pulse sequence without Nuclear Overhauser Enhancement and a pulse delay of 12 s; 10 000–20 000 transients and 32k data points were acquired over a frequency range of 25 kHz.

EPR Conditions. General EPR spectral parameters were as follows: scan range, 500 G for powders and 100 G for solutions; field set, 3275 G for PGA-amide powders, 3254 G for solutions, and 1875 G for the standard reference material (SRM); modulation amplitude, 0.5 G; microwave frequency, 9.1–9.15 GHz; modulation frequency, 100 kHz; microwave power, 6.32 mW; scan times, 4–16 min depending on concentration and gain levels; time constant, 0.032–0.128 s on a Varian Series E-109B spectrometer at 20 °C.

For solid-state spectra approximately 5 mg of sample, enough to fill the bottom of 3–4-mm quartz EPR tubes, was used. On top of the powder was placed a ruby "wafer" SRM (11.38 × 10^{15} Cr^{3+} spins) as recommended by the National Bureau of Standards.[24] The total volume occupied by both the powder and the SRM was well within the volume element for maximum signal response. First-derivative spectra were collected for each material and doubly integrated with proper base-line correction by standard computational methods. For the estimation of a spin–lattice relaxation parameter ($T_{1e^{\bullet}}$), samples were critically coupled so that no variation in detector current occurred as a function of microwave power; standard power saturation procedures were followed.[25] We have designated this parameter as $T_{1e^{\bullet}}$, as opposed to T_{1e}, since power saturation measurements are a function of both T_{1e} and T_{2e}; the calculation of T_{2e} is difficult for solids without time domain capabilities. Our estimation of relative T_{2e}, used
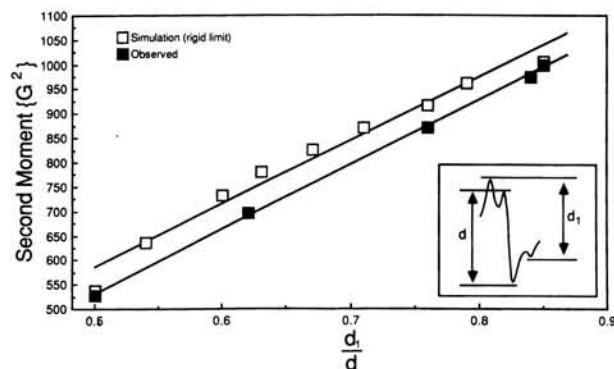

Figure 1. Relationship between the second moment ($\langle H^2 \rangle_{av}$) derived by numerical integrations[25] for both experimental (closed squares) and computer-generated first-derivative spectra (open squares) and the line width parameter $\{d_1/d\}$.[27] The ratio, d_1/d, is defined in the inset figure.

for the estimation of $T_{1e^{\bullet}}$, was calculated from computer-generated spectral line widths by standard procedures[26] ($g_{xx} = 2.0095$, $g_{yy} = 2.0059$, $g_{zz} = 2.0022$; $A_{xx} = 6.4$, $A_{yy} = 4.5$, $A_{zz} = 35.5$ G; $\Delta H_{pp} = 4$-14G). The relation between line width and T_2 is given by

$$1/T_2 = \frac{3^{1/2}\gamma}{2}\Delta H_{pp} \qquad (1)$$

In eq 1,[25] ΔH_{pp} is the line width; ΔH_{pp} was changed to produce spectra with various second moments ($\langle H^2 \rangle_{av}$; Figure 1). In this work, $T_{1e^{\bullet}}$ is reported in arbitrary units because of the inaccuracies involved in estimating absolute values of T_{2e} and, hence, T_{1e}. $\langle H^2 \rangle_{av}$'s for both computer simulations and experimental spectra were calculated by the method of even moments:[25]

$$\langle H^2 \rangle_{av} = \frac{\Delta H}{\Delta H^2 \sum\limits_{j=1}^{m}\sum\limits_{i=1}^{j}\left\{\frac{\delta I(H)}{\delta H}\right\}_i}\sum\limits_{j=1}^{m}\sum\limits_{i=1}^{j}(H_j - H_0)^2\left\{\frac{\delta I(H)}{\delta H}\right\}_i \qquad (2)$$

In eq 2, $\{\delta I(H)/\delta H\}_i$ represents the relative amplitude of each first-derivative data point, ΔH the magnetic field interval (0.67 G) between data points, and H_0 the magnetic field value where the double integral was $^{1}/_{2}$ maximum (e.g., the center of the spectrum). $\langle H^2 \rangle_{av}$'s (Figure 1) were quite linear with respect to the line-width parameter, $\{d_1/d\}$,[27] which is the amplitude between the low field first-derivative maximum and the high-field minimum divided by the total amplitude of the central hyperfine line (see inset in Figure 1). The calculation of $\langle H^2 \rangle_{av}$'s by numerical integrations could be problematic[28] due to cutoff errors since, for true Lorentzian lines, the spectral wings extend to infinity. Such cutoff errors in our case are lessened by the fact that the most broadened spectra were collected with a large scan range (500 G). However, these data (Figure 1) illustrate that this potential error was at least consistent relative to $\{d_1/d\}$ for both experimental and computer-derived EPR spectra.

Nearest-neighbor distance parameter (d; eq 3) calculations have been described previously:[6,7,27,29,30]

$$\langle H^2 \rangle_{av} = \frac{3}{5}g^4\beta^4h^{-2}S(S + 1)\frac{\kappa^2}{d^6} \qquad (3)$$

As shown before,[7] changes in $\langle H^2 \rangle_{av}$, which are related to line width, cannot be attributed entirely to changes in d; this is true because, as the lattice fills with paramagnetic centers, an increase in the number of near-neighbor spin–spin interactions ($\sim\kappa^2$) will occur. κ is assumed to be approximately 1 (dimer interactions only) at $d = 12$ Å (Figure 3) where $\langle H^2 \rangle_{av}$'s decreased sharply. Assuming d remains at 12 Å, κ values may be calculated for higher concentrations of the nitroxylamide; alternatively, κ may be assumed to be 1 and d calculated based on dimer interactions only.

For solution spectra, approximately 10 mg of powder was dissolved in 200 μL of H$_2$O and loaded into capillary tubes. Solution first-derivative spectra were collected with 6.32-mW microwave power; no line-width effects were observed when microwave powers were as high as 12 mW. Solution correlation times (τ_c, quadratic with respect to the nitrogen spin quantum
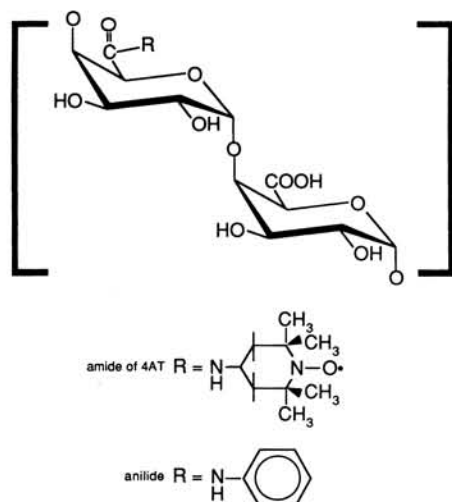
Figure 2. Chemical structures for the acid, anilide, and 4AT-amide derivatives of a polygalacturonan.
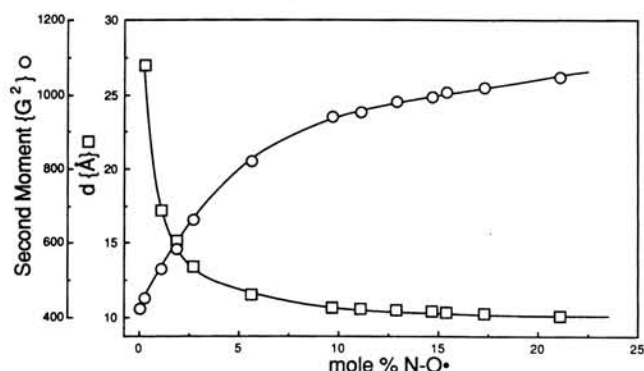

Figure 3. Dependency of calculated dimer-only nearest-neighbor distance parameters (d) and second moments ($\langle H^2 \rangle_{av}$) on the mole percentage of polygalacturonate carboxyl groups reacted to form the amide of 4AT. Square symbols represent d and circles $\langle H^2 \rangle_{av}$. The PGA-amides of 4AT reacted in the solid state (Me$_2$SO:H$_2$O, 50% v/v) are represented by the following data points: 21.1, 12.9, and 2.7 mol % nitroxylamide. All the remaining samples were reacted in H$_2$O.

number) were calculated according to a method described previously.[31] For certain representative samples, the pH was increased to approximately 9–10 with NaOH and the sample subsequently heated. No decrease in τ_c was noted, indicating that the amides of PGA were stable with respect to β-elimination. Our experimental solution spectra qualitatively resemble those reported previously[32] for spin-labeled xylans.

Results and Discussion

For this work we have chosen the reaction of a carbodiimide derivative of PGA with a paramagnetic amine nucleophile as a means to study the physiochemical properties of PGA by spin-label methods.[17,19] This reaction specifically labels acid sugar polymers at the C-6 position (Figure 2) because only carboxyl functional groups[19-21] can form carbodiimide (EDC) activated esters or inter/intramolecular lactones[22] which then react with the primary amine group of 4AT to produce the amides of PGA (Table I and Figure 2).

However, upon reaction and subsequent dialysis, we observed a significant degree of line broadening in partially reacted PGA-amide powders (Figure 3), indicating that the nitroxylamides were occurring in relatively small blocks or linear arrays. If this is true, the first-derivative PGA-amide powder patterns should display a degree of EPR line broadening which is close to the average intracarboxyl distance.[6,7,27,28,30,33-35] Dimer-only interspin distances (d, [N-O·] where $\kappa \sim 1$) were estimated to be about 12 Å from
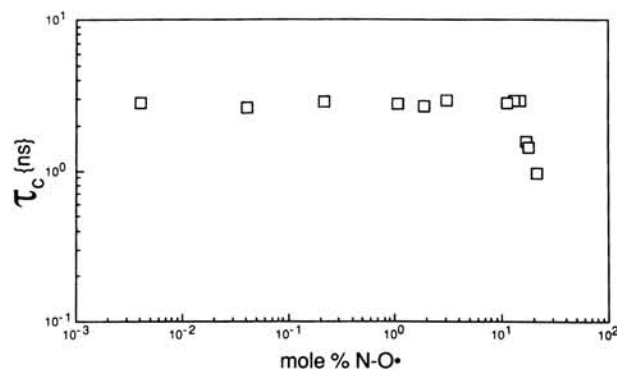

Figure 4. Dependency of solution correlation time (τ_c) on the mole percentage of the nitroxylamide (mol % N-O·) in labeled polygalacturonate at 20 °C. The average τ_c between 0.04% and 10% was 2.85 ± 0.05 ns (±S.E.). The change in τ_c above 10 mol % could be related to the nitroxyl's ability to cause disaggregation of the polymer and therefore increase the N-O·'s freedom to rotate about the amide bond.

$\langle H^2 \rangle_{av}$ and is 1–1.5× the within-chain carboxyl group distance.[14,36] This is a remarkable finding at pH 4.75 (pK_a = 3.23[36]) since the reaction favors the anionic carboxyl form[22] and therefore numerous reactive sites were presumably available. It is possible that our d values are somewhat large due to exchange narrowing;[34,35,37] however, this effect should be small at distances near 12 Å. These findings argue for either a sequential nucleophilic attack of 4AT on contiguously "activated" carboxyl functional groups[21] or the formation of linear arrays of carbodiimide-induced inter/intramolecular lactones[22] which can form amides with 4AT's primary amine. The nitroxyl-amide powder patterns narrowed rapidly (Figure 3) below 6.25 mol % bound N-O· where they presumably experienced fewer dipolar interactions from near-neighbor spins. The spin concentration where dimer interactions primarily occurred ($\kappa = 1$ where d begins to increase nonlinearly[7]) was on the order of 5–6 mol % nitroxylamide, indicating that 1 spin–spin dimer was present for every 30–40 monomer units which is close to the average degree of polymerization for these polymers[38] and argues for a uniform distribution of spin pairs at these low concentrations. There was also observed only small changes (ca. 5%) in either $\langle H^2 \rangle_{av}$ or $\{d_1/d\}$ as the available sites were filled, indicating that the derivatized regions did not tend to preferentially aggregate together in extended arrays.

Other data (Figure 4) indicate that spin-labeled polymer solutions became less ordered as EDC-activated sites filled since the solution correlation times (τ_c), which were relatively constant between 0.004 and 10 mol % (2.85 ± 0.05 ns), diminished once a certain threshold spin concentration was reached (ca. 10 mol %). This observation could be interpreted as the result of PGA complex dissociation due to structural constraints brought on by the addition of the relatively large nitroxyl moiety. This hypothesis is also supported by the fact that the derivatized PGA was more soluble in water than normal PGA under similar conditions. This interpretation also seems reasonable since much of the available experimental evidence concerning the physicochemical properties of similar polymer species indicates that these compounds can be highly extended[4,38,39] in aqueous solution due to aggregational effects.

If the line broadening shown in Figure 3 resulted from an internitroxyl spatial effect,[6] various dipolar interaction-related parameters ($\langle H^2 \rangle_{av}$, $\{d_1/d\}$ and an electron spin–lattice relaxation parameter or $T_{1e·}$) should approach those of nitroxyl groups randomly spaced in a solid lattice upon competitive reaction with a nonparamagnetic amine

Table II
Relative Intensity and ^{13}C Chemical Shift (δ) of Anilide Derivatives of Poly(galacturonic acid) after Reduction, Mild Acid Hydrolysis, and Dialysis (1000 Molecular Weight Cutoff Tubing)

		experiment (rel intensity)					
δ, ppm	functional group	I	II	III	IV	$\bar{X} \pm$ S.E.	
168.77 ± 0.09	amide C=O	1.00^a	0.84^b	0.93^a	0.81^c	0.89 ± 0.05	^{d} total amide
59.50 ± 0.42	reduced C-6	0.29	0.26	0.30	0.27	0.28 ± 0.01	$= 80.5\% \pm 0.12\%$
136.01 ± 0.96							
^{e}(2) 129.13 ± 0.44							
125.59 ± 0.15	aromatic C	5.30	6.70	6.36	5.58	5.98 ± 0.38	
(2) 121.94 ± 0.20							
99.64 ± 0.63	C-1	1.00	1.00	1.00	1.00		
77.39 ± 0.27							
71.17 ± 0.95	COH	4.49	3.74	4.00	3.73	3.99 ± 0.21	
(2) 68.85 ± 0.78							

^{a} Starting product = 25 mol % anilide; all signal intensities were normalized to the anomeric carbon resonance; all hydrolyzes were performed at 100 °C in 0.1 N H_2SO_4 for 0.5 h; solutions were neutralized with $BaCO_3$ which results in a $BaSO_4$ precipitate. ^{b} 31 mol % anilide. ^{c} 19 mol % anilide. ^{d} Assuming that the anilides react in sequential blocks, as predicted from the EPR data, the average degree of polymerization (DP) of these blocks would be approximately 9 with one galactosyl linkage on either terminal of the amide oligomer; thus, the predicted ratio of amide carbonyl to total anomeric carbon would be 9/11 or 0.82. ^{e} Chemical shifts preceded by (2) indicate the occurrence of two peaks which overlap.
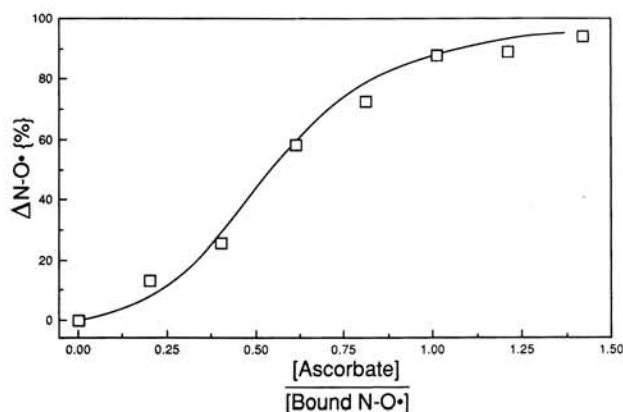

Figure 5. Dependency of the percent change in PGA-amide nitroxyl spin concentration (ΔN–O$^\bullet$) on the molar ratio of ascorbate to the original fixed spin concentration ([ascorbate]/[bound N–O$^\bullet$]). Prior to reduction, the sample had approximately 10% of the carboxyl groups reacted as the amide (highlighted square in Figure 7).

Figure 6. EPR spectra of the PGA-amide of 4AT as a function of lattice spacing. Both spectra contain a similar concentration of the nitroxyl spins (ca. 4 mol %) attached to PGA through amide groups. Spectrum B (original spin concentration ca. 9 mol %) resulted from partial ascorbate reduction in solution at 60 °C; unreacted ascorbate and dihydroascorbate were removed by dialysis.

(aniline) or by the partial reduction of the labeled lattice with ascorbate (Figures 5–7). Figure 5 demonstrates that the reaction of previously labeled PGA (spin concentration = 9.63 mol % N–O$^\bullet$) with ascorbate effectively reduced the nitroxylamide of PGA to its hydroxylamine form[40,41] at a near 100% efficiency as the ratio of ascorbate to bound [N–O$^\bullet$] approached 1.5. Differences in line widths were apparent when spectra (Figure 6) for polymer samples having a similar total spin concentration (ca. 4 mol %) but different spin spacings, induced by ascorbate reduction, were compared. Spectrum A is a typically broadened nitroxylamide at ca. 4 mol % N–O$^\bullet$; however, spectrum B, which originally had a spin concentration of 9.63 mol % spin label, upon partial reduction to 4 mol % with ascorbate resulted in a significant measure of line narrowing. This finding clearly indicates that the spins originally added in a spatially sequential fashion and that upon reduction, blocks of hydroxylamine disrupted the nitroxylamide spacing.

Electron spin–lattice relaxation times (T_{1e}) have been found to be related to interspin distances.[42,43] Regardless of the degree of dilution, the apparent spin–lattice relaxation time is dominated by the "local geometry"[43] around the free radical. If the nitroxylamide line broadening we have observed above is due to relatively short interspin distances, as we believe, the disruption of the PGA 4AT-amide lattice, through reduction or by competitive reac-
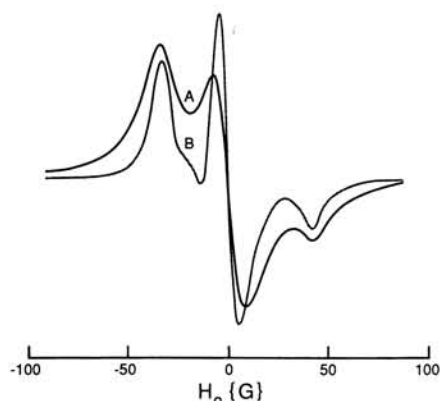
tions with both a paramagnetic and nonparamagnetic amine of similar size (e.g., aniline), should induce a corresponding increase in the relaxation time parameter (T_{1e^*}). The data in Figure 7 clearly illustrate this principle. Upon reducing part of the nitroxyl's spin or reacting PGA with both 4AT and aniline, in the presence of EDC, the effect on relative T_{1e^*} was substantial. The nitroxylamide T_{1e^*}'s increased by a factor of about 4 upon reduction with ascorbate to a total spin concentration of approximately 0.6 mol % N–O$^\bullet$ ([N–O$^\bullet$]/[N–OH + N–O$^\bullet$] = 0.055). The relaxation parameters for the aniline-reacted polymer were even more affected, indicating that aniline causes a greater spatial perturbation than by partially reducing the nitroxylamides of PGA. This observation is likely due to the fact that aniline has a smaller pK than 4AT and therefore more aniline molecules are available to react (e.g., in an unprotonated form); because of this, more anilide functional groups are covalently bound than reduced 4AT-amides and could result in greater nitroxylamide spacing.

Table II provides further evidence for the unusual sequential reaction which we propose. In these experiments, partially reacted propionate esters of PGA (19–31 mol % anilide) were first reduced[23] to their polygalactose anilide form followed by mild acid hydrolysis (0.1 N H_2SO_4; 0.5 h at 100 °C). If our sequential reaction hypothesis is true, then under conditions of mild acid hydrolysis the galactose regions should cleave preferentially leaving blocks of an-
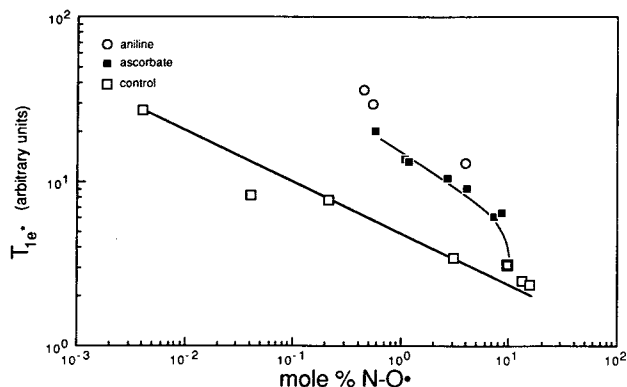
Figure 7. Relationship between covalently bound spin concentration and the electron spin–lattice relaxation time-related parameter (T_{1e*}, arbitrary units) relative to that predicted for the control, untreated PGA-amides of 4AT (open squares), competitively reacted (with aniline, open circles) amides of PGA, and partially ascorbate-reduced nitroxylamides (closed squares). Control data (no treatment) include the highlighted open square which represents the sample used for the ascorbate treatments (at. ~9 mol %); in the competitive reaction aniline was 30-, 10-, and 2-fold more concentrated than 4AT.

ilide with galactose occurring at, perhaps, terminal positions (uronosyl linkages are resistant to acid hydrolysis[44]). NMR results indicate that upon hydrolysis and subsequent dialysis (1000 molecular weight cutoff tubing) the anilide concentration of the oligomers increased 3-fold (e.g., from an average initial concentration of ~25 to a final concentration of ~81 mol % anilide). Assuming the aniline reacts to form sequential blocks of the anilide derivative, as predicted from the EPR results, the average DP of these regions would be approximately 9 with possibly one galactosyl linkage at the reducing and nonreducing terminals. Thus, the predicted average ratio of amide carbonyl to total anomeric carbon would be on the order of 9/11 or 0.82. The amide concentration calculated from the average of both 59.5 (reduced C-6) and 168.8 (amide carbonyl) ppm resonances indicated that the degree of amidation of these oligomers was ca. 81 mol % (Table II) which strongly supports the sequential reaction hypothesis.

Conclusions

Our results for spin–spin broadening, spin–lattice relaxation, and hydrolytic cleavage all are in accord with the hypothesis that the amide formation occurs in a sequential fashion. In addition, amidation of PGA with nitroxyl spin labels to 10% of total sites or greater is suggested to hinder the ability of the polymer to aggregate. Clearly, if this hypothesis is true, reaction at some initial site causes a change in the conformation or intermolecular coordination of these polymers such that near-neighbor sites react more readily, resulting in short linear arrays or intramolecular blocks of the paramagnetic amide. Coordination processes in these or similar compounds[4] have been used to explain the large excess enthalpy of proton dissociation over relatively small changes in degree of ionization observed for uronide-containing polymers. Other workers have also reported unusual aggregative behavior.[39] Perhaps the best evidence for coordination complexes in PGA-containing polymers is derived from size exclusion chromatography[38] which provides apparent molecular weights an order of magnitude larger than the true average molecular size. For PGA this is approximately 7000 daltons, as measured by reducing end group titration.[38] If true, the unusual aggregative properties of PGA-containing polymers could explain why changes in their apparent molecular weight[2,45–47] have been reported without a concomitant

increase in the activity of specific hydrolyases or changes in methyl esterification.[46] The presence of coordination complexes in native PGA-containing structures are important since they could potentiate sequential cation binding at coordinated sites along the polyanion's main chain.[6,7,46] Such coordination complexes could also result in the formation of sequential methyl ester arrays, as has been proposed before,[7] since the conformation of the polymer could affect the activity of the pertinent enzymes, possibly in a fashion similar to the EDC-mediated reaction reported herein.

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$$2\pi\nu_{mod} \cdot T_{1e} < 1.0$$

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