The Interaction of Biological Molecules with Heparin and Related Glycosaminoglycans. 1. Identification of a Specific Heparin Binding Site for Histamine

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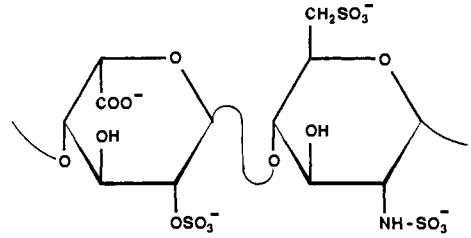
Abstract: The interaction of histamine with beef lung heparin has been characterized by ^1H and ^{13}C NMR spectroscopy. ^1H chemical shifts provide evidence that histamine forms a complex with heparin both at low pD where 2-*O*-sulfo-α-L-idopyranosyluronic acid (IdoA-2S) residues are in the carboxylic acid form and at higher pD where the carboxyl groups are deprotonated. At low pD, the chemical shift data are consistent with weak delocalized binding of the diprotonated form of histamine by the highly negatively charged polymer. However, as the pD is increased and the carboxylic acid groups are titrated, a binding region with a high affinity for diprotonated histamine is created. It is proposed that, in this binding site, the ammonium group of histamine is hydrogen-bonded to the carboxylate group of an IdoA-2S residue while the imidazolium ring is located in a region surrounded on three sides by the sulfamido group of a 2-deoxy-2-sulfamido-6-*O*-sulfo-α-D-glucopyranose ($GlcNSO_3$-6S) residue and the 2-*O*-sulfate and carboxylate groups of a second IdoA-2S residue. Evidence for site specific binding includes displacement of chemical shift titration curves to lower pD and increased shielding of specific heparin protons from the imidazole ring current. Similar ^1H NMR measurements on the heparin–imidazole and heparin–*N*-acetylhistamine systems indicate that the highly negatively charged binding site created by deprotonation of the carboxylic acid groups has a high affinity in general for positively charged imidazolium rings. Nuclear Overhauser enhancements obtained from two-dimensional NOESY spectra of heparin at pD 2.4 and 7.4 indicate that, when the carboxylic acid groups of heparin are titrated, its conformation changes by a small rotation around the (IdoA-2S)-(1→4)-($GlcNSO_3$-6S) glycosidic bond. This study provides evidence for the first time of the site specific binding of a biological molecule by the repeating (IdoA-2S)-(1→4)-($GlcNSO_3$-6S) disaccharide unit of heparin.

Introduction

Heparin and the related glycosaminoglycans dermatan sulfate, heparan sulfate, chondroitin-4-sulfate, and chondroitin-6-sulfate are highly negatively charged polysaccharides found in animal tissues. All are alternating copolymers of uronic acid (L-iduronic acid and/or D-glucuronic acid) and hexosamine (D-glucosamine or D-galactosamine) residues. Their high negative charge density results from the presence of ester sulfate and uronic acid carboxylate groups in the repeating disaccharide units. They are distinguished by their monosaccharide composition, by the position and configuration of their glycosidic linkages, and by the number and location of their sulfate substituents. Because of their high negative charge density, many biological molecules can bind to these glycosaminoglycans.[1-4] The binding of peptides, proteins, and other biological molecules by heparin is of particular interest, in view of its widespread therapeutic use as an anticoagulant and antithrombotic.[4-8]

The structure of heparin is largely accounted for by repeating sequences of the trisulfated disaccharide {(1→4)-*O*-(2-*O*-sulfo-α-L-idopyranosyluronic acid)-(1→4)-*O*-(2-deoxy-2-sulfamido-6-*O*-sulfo-α-D-glucopyranose)}. For example, this repeating disaccharide accounts for at least 85% of heparins from beef lung and about 75% of those from intestinal mucosa.[9,10] The two



monomers of the repeating disaccharide will be represented here by (IdoA-2S) and ($GlcNSO_3$-6S) and will be referred to as the I and A rings, respectively. Minor constituents, including β-D-glucopyranosyluronic acid and 2-deoxy-2-acetamido-6-*O*-sulfo-α-D-glucopyranosyl residues, are present in varying amounts, depending on the origin of the heparin.[11] It is well established that the anticoagulant activity of heparin is a result of the binding of antithrombin III to a unique pentasaccharide segment found in approximately one-third of the chains of mammalian heparins.[9]

Heparin is present in tissues as an insoluble complex with a basic, relatively small protein in the dense, membrane-enclosed granules of mast cells.[12] These granules, which represent ~50% of the total mast cell volume, act as a store for histamine;[13,14] it has been estimated that, if all the mast cell histamine were in solution in the total granule volume, the histamine concentration would be 0.42 M.[15] Although the nature of the binding of histamine in mast cell granules has not been established, evidence suggests that the heparin of the heparin–protein complex provides the binding sites.[16] Heparins from various tissues typically contain

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Table I. ^{13}C Chemical Shifts of Heparin[a,b]

carbon	heparin[c] pD 6.99	heparin[c] pD 2.00	$\Delta\delta$[e]	heparin and histamine[d] pD 6.02	heparin and histamine[d] pD 2.00	$\Delta\delta$[e]
A1	97.50	98.68	−1.18	97.87	98.93	−1.06
A2	58.77	58.71	0.06	58.77	58.76	0.01
A3	70.47	70.38[f]	0.09	70.61	70.40	0.21
A4	76.90[f]	77.06	−0.16	76.58	76.97	−0.39
A5	70.07	70.38[f]	−0.31	70.04	70.34	−0.30
A6	67.2[g]	67.1[g]	0.1	67.2[g]	67.1[g]	0.1
I1	100.16	100.08	0.08	99.80	99.89	−0.09
I2	76.82	76.09	0.73	76.26	75.86	0.40
I3	70.14	69.23	0.91	69.53	68.95	0.58
I4	76.90[f]	77.15	−0.25	77.00	77.30	−0.30
I5	70.31	68.68	1.63	69.81	68.50	1.31
I6	175.22	173.03	2.19			

[a] In D_2O, 37 °C. [b] In ppm relative to TMS. [c] Heparin disaccharide, 0.060 M. [d] Heparin disaccharide, 0.060 M, and histamine, 0.060 M. [e] $\Delta\delta = \delta_{pD\ 6.99(6.02)} - \delta_{pD\ 2.00}$. [f] Overlapping signals. [g] Less certainty due to multiple peaks.

3.0–3.5 anionic sites per disaccharide repeat unit.[11] In mast cell granules, there are 2.53 anionic sites available per heparin disaccharide repeat unit for binding the dyes acridine orange and methylene blue, and by analogy for binding histamine, which suggests that an *O*-sulfate is responsible for ionic binding of granule heparin to granule proteins, while the *N*-sulfate, the iduronate carboxylate, and a variable number of *O*-sulfate groups are available for binding dye or histamine molecules.[17]

At the intragranular pH (pH 5–6),[18,19] histamine in its diprotonated form is thought to be associated with the granule matrix by ionic interactions with the highly negatively charged heparin.[16] The carboxylate group of the iduronic acid residue is implicated from the pH dependence of the binding;[17] a model has been proposed in which an imidazole ring nitrogen is near the carboxylate group and the amino nitrogen is near an *N*-sulfate oxygen of the next residue.[20,21]

In a previous study, we demonstrated that histamine can be observed directly in intact mast cells and in intact mast cell granules by ^1H nuclear magnetic resonance spectroscopy.[15] We also found that the binding of histamine by heparin in solution can be detected through a change in the effect of pH on the chemical shifts of resonances for the histamine protons. In this paper, we report the results of more detailed studies in which we have identified by ^1H NMR a specific heparin binding site for the diprotonated form of histamine. Although the binding of antithrombin by a unique pentasaccharide segment of some heparin chains is well established,[9] this study provides evidence for the first time of the site specific binding of a biological molecule by the much more abundant repeating (IdoA-2S)-(1→4)-($GlcNSO_3$-6S) disaccharide unit of heparin.

Experimental Section

Beef lung heparin (sodium salt) (153 USP units/mg, 16 000–17 000 molecular weight), beef intestinal mucosal heparin (sodium salt) (172 USP units/mg, 10 000–12 000 molecular weight), the free base forms of *N*-acetylhistamine and imidazole, and histamine hydrochloride were obtained from Sigma Chemical Co. Histamine, 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, and methylamine hydrochloride were obtained from Aldrich Chemical Co.

NMR measurements were made on D_2O or H_2O solutions containing heparin, amine, or heparin plus amine. Heparin concentrations are expressed in terms of the concentration of the repeating disaccharide unit. pD was adjusted with concentrated solutions of DCl and NaOD in D_2O. pD was measured using microcombination pH electrodes whose response was calibrated with certified standard buffer solutions; pH meter readings were converted to pD using the equation $pD = pH_{meter\ reading} + 0.40$.[22]

^1H NMR spectra were measured at 400 and 500 MHz and ^{13}C spectra at 100 and 125 MHz with Varian XL-400 and VXR-500S NMR spectrometers. The H_2O resonance or the residual HDO resonance was suppressed either by selective presaturation for 1–2 s before application of the nonselective observation pulse or on the basis of differences in T_1 values of the H_2O or HDO resonance and the resonances of interest using the WEFT method.[23] ^1H chemical shifts were measured relative to the resonance for the methyl protons of internal *tert*-butyl alcohol (δ = 1.2365 ppm) or 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, δ = 0.0 ppm). ^{13}C chemical shifts were measured relative to internal dioxane (δ = 67.4 ppm).

Two-dimensional nuclear Overhauser effect (NOESY) spectra were measured at 500 MHz using the standard 2D NOESY pulse sequence.[24] All NOESY spectra were acquired at 37 °C with presaturation of the HDO signal (4.64 ppm at 37 °C) using a relaxation delay twice the longest T_1 (1.7 s) of the heparin protons. Conditions where NOESY cross peaks are still in the initial rate regime[25] were identified by measuring a series of NOESY spectra for a pD 7.9 solution containing 0.0098 M borine intestinal mucosal heparin and 0.15 M NaCl as a function of mixing time (0.025, 0.050, 0.075, 0.100, 0.125, and 0.150 s). A mixing time of 0.075 s was then used in the measurement of NOESY spectra for the following solutions of bovine lung heparin in D_2O: (1) 0.010 M heparin, 0.115 M NaCl, pD 2.4; (2) 0.010 M heparin, 0.115 M NaCl, pD 7.4; and (3) 0.0046 M heparin, 0.0042 M histamine, pD 4.9. NOESY spectra for solutions 1 and 2 were obtained using a 2600 Hz spectral width in F1 and F2, 2048 data points in F2, and 128 t_1 increments in F1; 64 transients were co-added at each t_1 increment in phase sensitive mode. The data were processed with zero-filling to 2048 points in F1, and negatively shifted sine bell functions were used to apodize the data in both F1 and F2. The NOESY spectrum for solution 3 was obtained with data acquisition and processing parameters as above, except a 2000 Hz spectral width was used in F1 and F2, and 32 transients were collected at each t_1 increment. Cross peak intensities are reported as the average and standard deviation of the ratios of the cross peak/diagonal peak volumes from three independent NOESY spectra measured for each sample.

^{13}C resonances were assigned using ^{13}C–^1H heteronuclear correlation spectroscopy. ^{13}C–^1H correlation spectra were measured by two-dimensional heteronuclear multiple quantum spectroscopy (HMQC) with ^1H detection for pD 2 and 7 solutions containing 0.060 M heparin and pD 2 and 6 solutions containing 0.060 M heparin plus 0.060 M histamine. Spectra were measured at 37 °C; 1024 × 128 data points were collected in phase sensitive mode and zero-filled to 2048 × 2048 (or 4096) data points. A Gaussian apodization function was applied to both dimensions prior to Fourier transformation. ^{13}C coupling was removed on the ^1H dimension by ^{13}C decoupling during acquisition. The reported chemical

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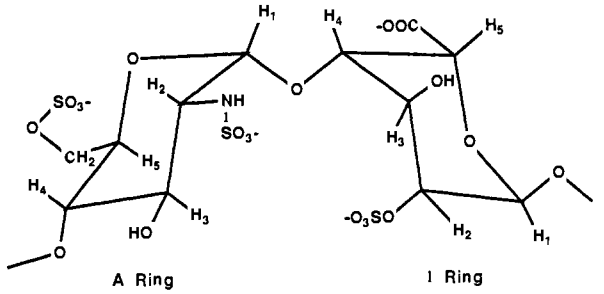
shifts were obtained from one-dimensional ^{13}C spectra measured with ^1H decoupling for the same samples.

Results

The binding of histamine by heparin was characterized by measuring ^1H and ^{13}C NMR spectra of solutions containing heparin, histamine, and heparin plus histamine. The ^1H and ^{13}C NMR spectra of heparin have been assigned by Perlin, Casu, and co-workers;[10] the ^1H assignments were confirmed and apparent ambiguities in the ^{13}C assignments were resolved in the present study. Because histamine is thought to bind to heparin by ionic interactions between anionic sites on heparin and the dipositive form of histamine,[16] spectra were measured as a function of pD to vary the protonation states, and thus the extent of binding, of the carboxylate groups of heparin and the imidazole and amino groups of histamine.

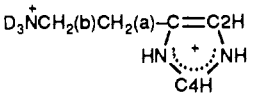
Heparin. The chemical shifts of resonances for the carbon-bonded protons of heparin are plotted versus pD in Figure 1, and ^{13}C chemical shift data for heparin solutions at pD 2 and 7 are reported in Table I. Assignments are given in the structure below, which shows the A and I rings in the $^4C_1$ and $^1C_4$ conformations, respectively.[9-11]



The data in Figure 1 show that the chemical shifts of most ^1H resonances of heparin change with pD over the pD range 2–6, where the carboxylic acid group on C-5 of the I ring is titrated. The I1, I3, I4, and I5 resonances shift upfield by 0.044, 0.106, 0.046, and 0.372 ppm, respectively, while the I2 resonance shifts downfield by 0.006 ppm. The changes in chemical shift are presumably due to through bond inductive effects, which decrease as the number of bonds between the carboxylic acid group and the carbon-bonded protons increases. The chemical shifts of five of the six I ring ^{13}C resonances also change upon titration of the carboxylic acid group, with four shifting downfield in the order I6 > I5 > I3 > I2. Although the A ring carbon atoms and protons are separated from the I ring carboxylate group by a minimum of four and five bonds, respectively, and thus changes in magnetic shielding due to through bond effects should be negligible, the resonance for the A1 carbon shifts upfield by 1.18 ppm while those for the A5 and A6, A6′ protons shift downfield by 0.130, 0.046, and 0.016 ppm. The large change in chemical shift of the resonance for the A5 proton has been interpreted by Perlin, Casu, and co-workers to indicate that the carboxyl group is in close proximity to the A5 proton of an adjacent A ring, and that there is a decrease in through space interactions between the carboxyl group and the A5 proton due to extension of the polyelectrolyte chain as a negative charge is created on the carboxyl group.[10]

As described below, deprotonation of the carboxylic acid group is found to be essential for the site specific binding of histamine by heparin. To elucidate the details of the change in conformation which accompanies deprotonation, NOE measurements were made at pD 2.4 and 7.4. Proton–proton coupling constants indicate that the conformations of individual residues are unaffected by titration of the carboxylic acid group,[10] and thus we have focused on the conformations across the glycosidic bonds. The NOESY spectrum of a pD 7.4 solution of heparin is shown in Figure 2, together with the normal one-dimensional spectrum plotted across the top and along the side. Interresidue NOE cross peaks are observed between anomeric proton A1 and protons I3 and I4 and between protons I1 and A4 and proton I1 and one of the A6 protons. NOEs are also observed between these same protons at pD 2.4. However, the cross sections in Figure 3 from the pD 2.4 and 7.4

Table II. Chemical Shifts of the Carbon-Bonded Protons of Histamine[a]



pD	δ_{C2H}	δ_{C4H}	δ_{CH_2}	δ_{CH_2}
2.89	8.666	7.388	3.344	3.160
8.60	7.726	7.038	3.261	2.954
12.36	7.670	6.918	2.841	7.699

[a] In ppm, relative to DSS.

NOESY spectra show that the magnitudes of the A1–I3 and A1–I4 NOEs are strongly pD dependent; the A1–I3 NOE increases from 0.027 ± 0.002 to 0.052 ± 0.002 when the pD is increased from 2.4 to 7.4, while the A1–I4 NOE decreases from 0.094 ± 0.004 to 0.061 ± 0.006. The I1–A4 NOE is essentially the same at pD 2.4 and 7.4, 0.050 ± 0.002 and 0.062 ± 0.007, respectively. The changes in the A1–I4 and A1–I3 NOEs as the pD is increased from 2.4 to 7.4 indicate that, as the carboxylic acid group is titrated, there is a change in conformation across the (GlcNSO$_3$-6S)-(1→4)-(IdoA-2S) glycosidic bond which increases the distance between protons A1 and I4 and decreases that between A1 and I3. Indeed, the NOEs suggest that proton A1 is approximately equidistant from protons I3 and I4 at pD 7.4. The anomalous large upfield shift of the resonance for the A1 carbon upon titration of the carboxylic acid group (Table I) provides additional evidence for a change in conformation across the (GlcNSO$_3$-6S)-(1→4)-(IdoA-2S) glycosidic bond. The similarity of the I1–A4 NOEs at pD 2.4 and 7.4 suggests that the conformation across the (IdoA-2S)-(1→4)-(GlcNSO$_3$-6S) glycosidic bond does not change upon titration of the carboxylic acid group.

Space-filling molecular models show that, upon rotation around the (GlcNSO$_3$-6S)-(1→4)-(IdoA-2S) glycosidic bond from a conformation in which the A1 proton is closer to I4 to one in which it is approximately equidistant between I3 and I4, the carboxylate group of the I ring moves closer to and in a position directly over the A5 proton. Thus, the NOE results suggest that the downfield shift of the A5 resonance (Figure 1) is due to deshielding by the anisotropic carboxylate group as a result of a change in conformation across the (GlcNSO$_3$-6S)-(1→4)-(IdoA-2S) glycosidic bond, and not to an increase in distance between the A5 proton and the carboxylate group, as suggested previously.[10]

Histamine. The chemical shifts of the carbon-bonded protons of histamine are pD dependent, changing over the pD ranges where the imidazolium and ammonium groups are titrated. The chemical shifts at pD values where histamine is present predominantly in the H_2A^{2+}, HA^+, and A forms are listed in Table II. As the pD is increased from 2.89 to 8.60 and the imidazolium group is titrated, the chemical shifts change in the order C2H > C4H > CH_2a > CH_2b. The chemical shift changes which accompany titration of the ammonium group are in the order CH_2b > CH_2a > C4H > C2H.

Heparin–Histamine. The pD dependence of the chemical shifts of the carbon-bonded protons of heparin and histamine is different in solutions containing both heparin and histamine. Exchange averaged resonances are observed for both heparin and histamine, indicating fast exchange of heparin and histamine between the free and bound forms. The chemical shifts of those heparin resonances which change by >0.1 ppm in the presence of histamine are plotted as a function of pD in Figure 4. Chemical shift titration curves A and B are for the I5 and A3 protons of heparin alone while curves C and D are for the same protons in a solution containing equimolar concentrations of histamine and heparin disaccharide. Also shown are chemical shift titration curves for the C2H proton of histamine; curve E is for the C2H proton of histamine alone while curve F is for the same proton in a solution containing equimolar concentrations of histamine and heparin.

In the presence of histamine, the chemical shift titration curve for the I5 proton of heparin is displaced to lower pD while that

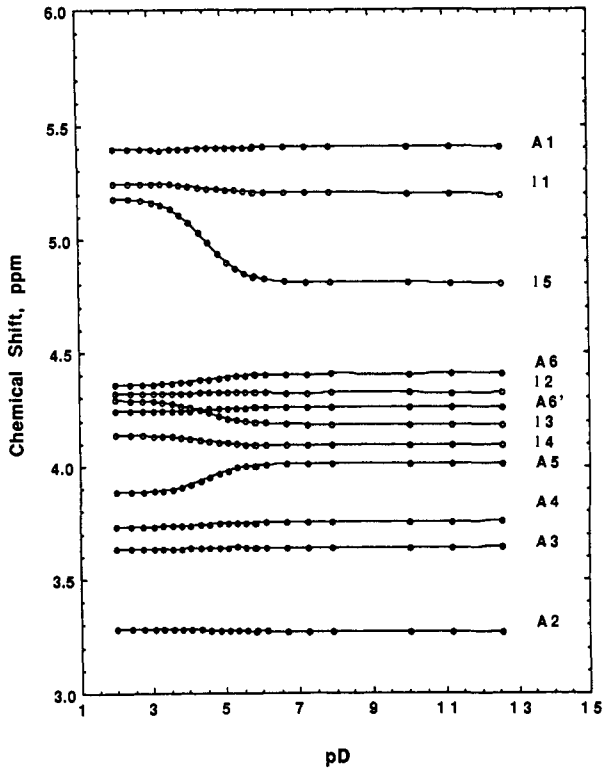


Figure 1. Chemical shifts of the carbon-bonded protons of 0.005 M bovine lung heparin in D_2O solution (0.005 M NaCl) as a function of pD. Assignments are given in the text. pD and ^1H NMR measurements were made at 25 °C. The HDO resonance was eliminated by the WEFT method.[23] The solid lines in this figure and in Figures 4–7 are drawn to connect experimental data points and have no theoretical significance.

for C2H of histamine is displaced to higher pD. These changes are consistent with formation of a heparin–histamine complex as described by the following equilibria:

$$HepCO_2H \rightleftharpoons HepCO_2^- + H^+ \quad (1)$$

$$HepCO_2^- + H_2A^{2+} \rightleftharpoons HepCO_2^-{-}H_2A^{2+} \quad (2)$$

$$H_2A^{2+} \rightleftharpoons HA^+ + H^+ \quad (3)$$

Formation of the $HepCO_2^-{-}H_2A^{2+}$ complex has the effect of shifting the deprotonation equilibrium for $HepCO_2H$ to the right, and thus displacing the I5 chemical shift titration curve to lower pD, while shifting the H_2A^{2+} deprotonation equilibrium to the left and displacing the C2H titration curve to higher pD.

Perhaps the most surprising result in Figure 4 is the large change in chemical shift of the resonance for the A3 proton. In the absence of histamine, the chemical shift of the A3 resonance is essentially independent of pD. However, in the presence of histamine, the resonance shifts upfield over the pD region where the carboxylic acid group is titrated and the $HepCO_2^-{-}H_2A^{2+}$ complex forms. The upfield shift reaches a maximum of 0.348 ppm at pD 6.13. As the pD is increased further and the imidazolium group titrated (titration curve F in Figure 4), the chemical shift of the A3 resonance moves back toward that of heparin in the absence of histamine, indicating dissociation of the $HepCO_2^-{-}H_2A^{2+}$ complex. Other A ring resonances also shift upfield in the presence of histamine, but the shifts are considerably smaller. For example, at pD 6.13, the A1, A2, A4, A5, and A6 resonances are shifted upfield by 0.078, 0.084, 0.088, 0.044, and 0.040 ppm, respectively. The shape of the chemical shift versus pD curve for the A3 resonance at pD >9 also suggests some binding of the monoprotonated form of histamine.

$$HepCO_2^- + HA^+ \rightleftharpoons HepCO_2^-{-}HA^+ \quad (4)$$

To determine if the conformation of heparin changes upon complexation of histamine, 2D NOESY spectra were measured for a D_2O solution containing 0.0046 M heparin and 0.0042 M histamine at pD 4.9. Cross sections taken at the I1 and A1 chemical shifts are plotted in Figure 3. The relative intensities of the A1–I3 and A1–I4 NOE cross peaks (0.052 ± 0.009 and 0.108 ± 0.004, respectively) suggest that, even though the carboxylic acid group is essentially completely titrated at pD 4.9 in the presence of histamine (Figure 4), the conformation across the $GlcNSO_3$-6S-(1→4)-IdoA-2S glycosidic bond is more similar to that of free heparin at pD 2.4. The intensity of the I1–A4 NOE

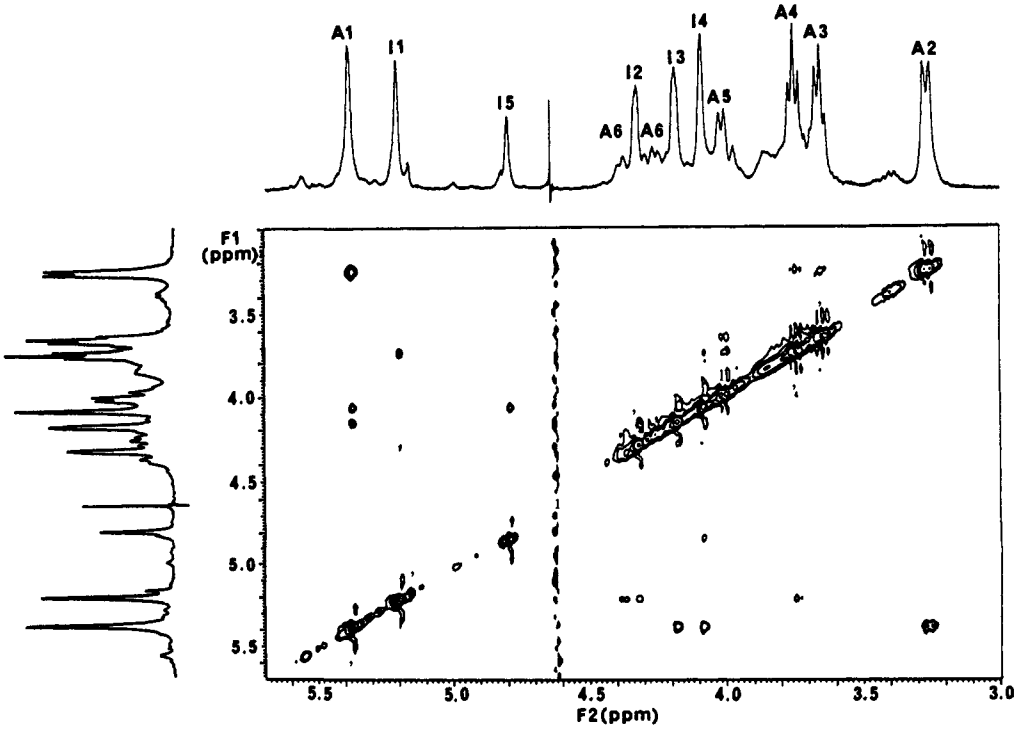


Figure 2. Two-dimensional NOESY spectrum of a pD 7.4 solution containing 0.010 M heparin and 0.115 M NaCl. The normal single pulse spectrum is plotted across the top and along the side. Resonance assignments are given in the text. Both 1D and 2D spectra were measured at 37 °C in order to shift the HDO resonance to 4.64 ppm. The HDO resonance was suppressed by presaturation.

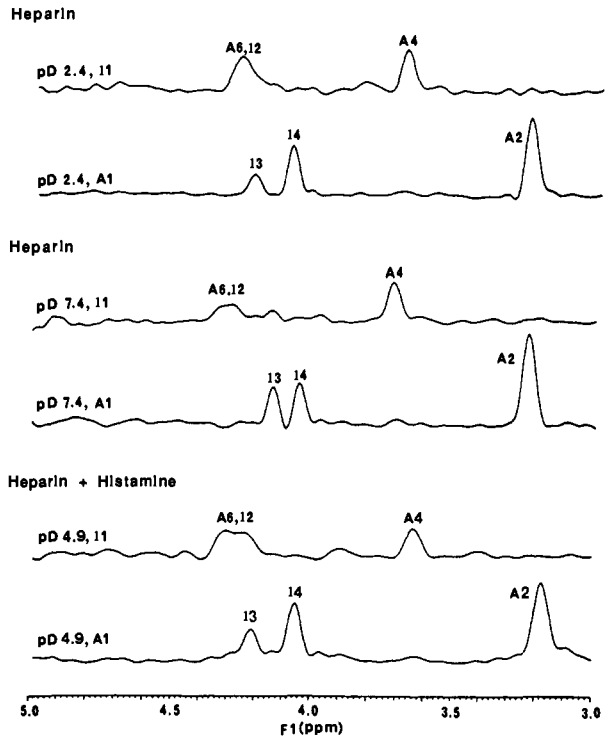


Figure 3. Cross sections from NOESY spectra of pD 2.4 and 7.4 solutions containing 0.010 M heparin + 0.11 M NaCl and a pD 4.9 solution containing 0.0046 M heparin and 0.0042 M histamine. Cross sections were taken at the chemical shifts of the I1 and A1 anomeric protons on the F2 axis.

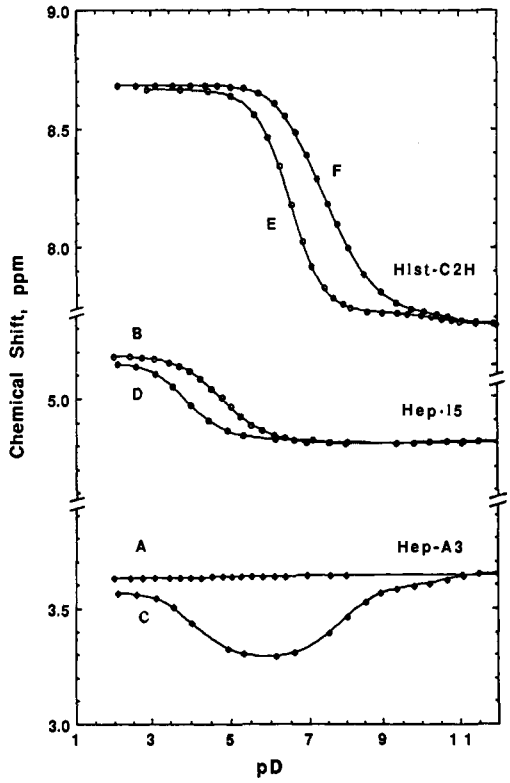


Figure 4. Chemical shifts of resonances for heparin A3 and I5 protons and the histamine C2H proton as a function of pD. Curves A and B are for 0.005 M heparin + 0.005 M NaCl; curves C, D, and F are for 0.025 M heparin + 0.025 M histamine; and curve E is for 0.005 M histamine + 0.018 M NaCl. Measurements were made at 25 °C and the HDO resonance was suppressed with the WEFT method.[23]

cross peak is essentially the same as in the absence of histamine (0.046 ± 0.012), which indicates no change in conformation across the IdoA-2S-(1→4)-GlcNSO$_3$-6S glycosidic bond.

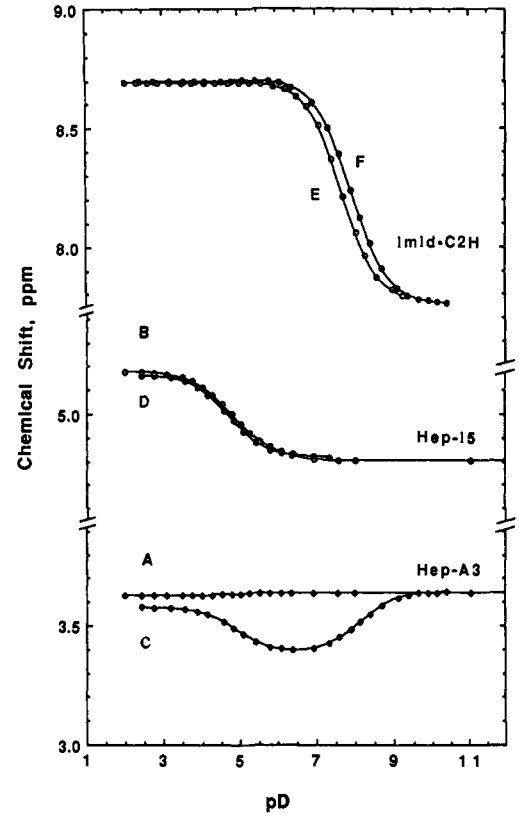


Figure 5. Chemical shifts of resonances for the heparin A3 and I5 protons and the imidazole C2H proton as a function of pD. Curves A and B are for 0.005 M heparin + 0.005 M NaCl; curves C, D, and F are for a solution of 0.025 M heparin + 0.025 M imidazole; and curve E is for 0.005 M imidazole + 0.018 M NaCl.

Heparin–Imidazole. To elucidate the nature of the binding of histamine by heparin, similar ^{1}H NMR measurements were made on solutions containing heparin and selected model compounds. Chemical shift data for the heparin–imidazole system are plotted in Figure 5. The pD dependence of the chemical shift of the resonance for the I5 proton is identical in the absence (curve B) and presence (curve D) of imidazole, which indicates no change in the apparent acidity of the I ring carboxylic acid group in the presence of imidazole. However, displacement of the chemical shift titration curve for the imidazole C2H proton to higher pD indicates that the imidazolium ion is complexed by heparin. The displacement is less than that observed for the heparin–histamine system, which suggests that the extent of complex formation is less than in the heparin–histamine system. The upfield shift of the A3 resonance also indicates complex formation. Although the chemical shift titration curves for the I5 proton suggest that the carboxylate group is not directly involved in binding the imidazolium ion, the fact that the upfield shift of the A3 resonance and titration of the carboxylic acid group take place over the same pD range indicates that titration of the carboxylic acid group is necessary for formation of the heparin–imidazolium ion complex. Other A-ring resonances also shift upfield but by much smaller amounts; e.g., at pD 6.4 where maximum binding occurs, the A1–A6 resonances shift upfield by 0.044, 0.060, 0.236, 0.062, 0.034, and 0.022 ppm. The chemical shift of the A6′ resonance and all the I-ring resonances are essentially unaffected by the complexation. The heparin–imidazolium ion complex dissociates when the imidazolium ion is deprotonated, as evidenced by the simultaneous return of the A3 resonance to its chemical shift in the absence of imidazole and the change in chemical shift of C2H over the pD range 6–9.

Heparin–Methylamine. Chemical shift data for the heparin–methylamine system are presented in Figure 6. In the presence of methylamine, the chemical shift titration curve for the I5 proton is displaced slightly to lower pD, corresponding to a small increase in the apparent acidity of the carboxylic acid group, while in the

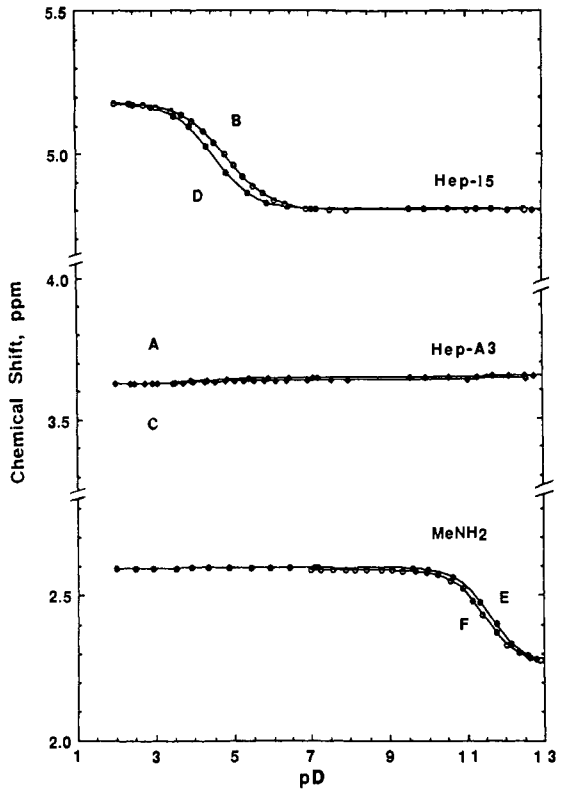


Figure 6. Chemical shifts of resonances for the heparin A3 and I5 protons and methylamine CH_3 protons as a function of pD. Curves A and B are for 0.005 M heparin + 0.005 M NaCl; curves, C, D, and F are for 0.025 M heparin + 0.025 M methylamine; and curve E is for 0.005 M methylamine + 0.018 M NaCl.

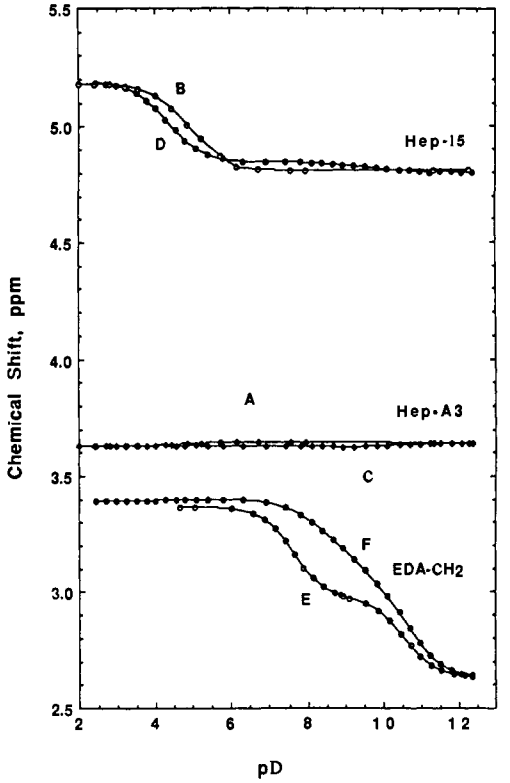


Figure 7. Chemical shifts of resonances for the heparin A3 and I5 protons and the CH_2 protons of 1,2-ethanediamine (EDA) as a function of pD. Curves A and B are for 0.005 M heparin; curves C, D, and F are for 0.005 M heparin + 0.005 M EDA; and curve E is for 0.005 M EDA = 0.018 M NaCl.

presence of heparin, the chemical shift titration curve for the methylamine protons is displaced to higher pD, corresponding to a decrease in the apparent acidity of the methylammonium ion. These data are consistent with the formation of a $HepCO_2^-$–methylammonium ion complex. The chemical shift of the A3 proton is unaffected by the presence of methylamine, which indicates that the nature of the complex formed is different from the heparin–histamine and heparin–imidazole complexes.

Heparin–*N*-Acetylhistamine. The chemical shifts of the carbon-bonded protons of heparin and *N*-acetylhistamine were measured as a function of pD for a solution containing both heparin and *N*-acetylhistamine at a concentration of 0.005 M (data not shown). The presence of *N*-acetylhistamine has no significant effect on the chemical shift titration curve for the I5 proton of heparin. However, the resonance for the A3 proton shifts upfield as the carboxylic acid group is titrated, indicating formation of a heparin–*N*-acetylhistamine complex. At higher pD, the chemical shift titration curve for the imidazole group of *N*-acetylhistamine is displaced to higher pD, and the A3 resonance moves back to its chemical shift in the absence of *N*-acetylhistamine. These results are identical with those observed for the heparin–imidazole system and indicate formation of a heparin–*N*-acetylhistamine complex in which heparin binds the imidazolium ring.

Heparin–α,ω-Diamines. The diprotonated form of histamine apparently forms a stronger complex with heparin than do either the methylammonium ion or the imidazolium ion, as evidenced by the relative displacements of the chemical shift titration curve for the I5 resonance to lower pD in the presence of histamine and methylamine and the relative displacements of the chemical shift titration curves for the C2H resonances of histamine and imidazole to higher pD in the presence of heparin. To determine if the extent of binding is larger for other diprotonated small molecules, the binding of 1,2-ethanediamine (EDA), 1,3-propanediamine (PDA), and 1,4-butanediamine (BDA) by heparin was studied. Chemical shift data are presented in Figure 7 for the heparin–EDA system. In the presence of EDA, the chemical shift titration curve for the I5 resonance is displaced to lower pD, and the displacement is larger than that observed in the presence of methylammonium ion but less than in the presence of histamine, which suggests the extent of binding to be histamine > EDA > methylamine. Additional evidence for the binding order EDA > methylamine is the larger displacement of the chemical shift titration curve for EDA to higher pD. These results suggest that both ammonium groups are involved in the binding of EDA to heparin. It is of interest to note that, as in the heparin–methylamine system, the chemical shift of the A3 resonance of heparin is not affected by binding of EDA. Similar results were obtained for the heparin–DAP and heparin–DAB systems; however, the apparent extent of binding is slightly less than observed for the heparin–EDA system.

Discussion

Heparin is a highly negatively charged polymer, with a fraction of its negative charge neutralized by bound counterions.[26-35] As such, it behaves as a polyelectrolyte.[36] It is well established that, in typical cases, counterions are not bound to polyelectrolytes by covalent bonds. Rather, in Manning's counterion condensation theory for polyelectrolyte solutions, the polyelectrolyte is considered

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to be a uniformily charged line of infinite length, interacting with a solution of mobile, bound counterions.[36,37] The bound counterions are contained in a cylindrical condensation volume which exists around the polyelectrolyte. They are considered to be in the fully hydrated state characteristic of a purely aqueous environment and to be bound territorially, rather than site specifically, i.e., to have unrestricted freedom of motion along the length of the polyanion within the condensation volume. The binding mechanism for territorially bound counterions involves only long-range electrostatic interactions between counterion and ionic sites on the polyelectrolyte. For example, evidence indicates that Mg^{2+} ions are territorially bound to heparin,[30,33] whereas there is conflicting evidence regarding the nature of the binding of Ca^{2+} and Zn^{2+}.[27-35]

Taken together, the chemical shift data in Figures 4–7 provide evidence that histamine is bound by heparin. The small upfield shifts of the resonances for the A3 and I5 protons in the presence of histamine at low pD (Figure 4) indicate a small amount of binding when histamine is in the diprotonated form and the I ring carboxyl group is in the carboxylic acid form. Similar small changes in chemical shift are also observed for other heparin protons, which suggests the binding at low pD is territorial, rather than site specific.

The chemical shift data indicate, however, that binding of histamine by the carboxylate form of heparin is site specific. The displacement of the chemical shift titration curve for the I5 resonance to lower pD is consistent with a specific interaction between the ammonium group of histamine and the carboxylate group of the I ring which competes with deuterium ions for the carboxylate group, while the upfield displacement of the A3 resonance indicates a specific interaction of the imidazolium group with the A ring. We propose that, when the I ring carboxylic acid group is titrated, a binding site with a high affinity for the positively charged imidazolium ring is created. In this binding site, the imidazolium ring is located above the A3 proton and thus the A3 resonance shifts upfield due to ring current effects.[38] Space filling molecular models, with the A and I rings in the $^4C_1$ and $^1C_4$ conformations, respectively,[39] suggest that the ammonium group of bound H_2A^{2+} is hydrogen-bonded to the carboxylate group of the first I ring of an (IdoA-2S)-(1→4)-($GlcNSO_3$-6S)-(1→4)-(IdoA-2S) sequence while the imidazolium ring is located in a pocket surrounded by three anionic groups, the sulfamido group of the A ring and the 2-*O*-sulfate and carboxylate groups of the next I ring. The small ring current effect on the chemical shift of the A3 resonance at pD >9 suggests that binding of the monoprotonated form of histamine also is site specific.

A 1:1 histamine–heparin disaccharide stoichiometry has been determined by equilibrium dialysis for the histamine–heparin complex.[40] It also has been determined by X-ray fiber diffraction studies that crystalline heparin exists in a helical structure.[41] A space-filling molecular model of helical heparin shows that the proposed histamine binding site, with the carboxylate group of each I ring being part of the binding cavity for the imidazolium ring of one histamine and hydrogen-bonded to the ammonium group of the histamine in the next binding site, is consistent with a 1:1 histamine–heparin disaccharide stoichiometry.

The chemical shift data in Figures 5 and 6 for the heparin–imidazole and heparin–methylamine systems support this model for the binding of histamine by heparin. The data indicate that ammonium and imidazole groups can bind independently to the carboxylate group and A-ring imidazolium binding sites, respectively. The absence of any change in the chemical shifts of the I-ring resonances in the presence of imidazole provides additional evidence that the bound imidazolium ion is localized near the A ring. The small upfield shifts of resonances for the A1, A2, A4, A5, and A6 protons in the presence of bound imidazolium ion, histamine, and *N*-acetylhistamine suggest that the bound imidazolium ion or the imidazolium groups of histamine or *N*-acetylhistamine are sufficiently close to these protons part of the time to cause increased shielding by ring current effects; however, the much larger upfield shift of the resonance for the A3 proton indicates that the bound imidazolium groups are located precominantly in the proposed binding site. The magntidues of the displacement of chemcial shift titration curves to higher or lower pD in the heparin–imidazole and heparin–methylamine systems, relative to those for the heparin–histamine system, show that the binding of histamine is stronger.

The identical chemical shift titration curves for the I5 resonance in the absence and presence of imidazole indicates that the imidazolium ion does not interact with the carboxylate group in a way which competes with deuteration; however, site specific binding of the imidazolium ion is dependent on conversion of the carboxylic acid group to the carboxylate anion, as evidenced by the change in chemical shift of the A3 resonance over the pD range where the carboxyl group is titrated. The intensities of the A1–I3 and A1–I4 NOESY cross peaks for free heparin at pD 2.4 and 7.4 (Figure 3) and the chemical shift of the A1 carbon at pD 2 and 7 (Table I) indicate that the conformation of heparin changes by a small rotation around the ($GlcNSO_3$-6S)-(1→4)-(IdoA-2S) glycosidic bond upon titration of the carboxylic acid group. However, the relative magnitudes of the A1–I3 and A1–I4 NOEs of histamine-complexed heparin are closer to those of the carboxylic acid form of heparin, which suggests that the dependence of histamine and imidazole binding on titration of the carboxylic acid group is a result of the formation of a binding pocket surrounded on three sides by anionic groups and not a conformational change across the ($GlcNSO_3$-6S)-(1→4)-(IdoA-2S) glycosidic bond. A space filling molecular model shows that a small rotation around the ($GlcNSO_3$-6S)-(1→4)-(IdoA-2S) glycosidic bond to decrease the distance between the A1 and I4 protons and increase that between the A1 and I3 protons has the effect of increasing the size of the proposed binding pocket for the imidazolium ring.

Conclusion

The NMR results presented here indicate that histamine is bound by heparin, with the nature and extent of the binding being very strongly pD dependent. Starting at low pD where the I ring carboxyl group is in the carboxylic acid form, some histamine is bound territorially in the condensation volume around heparin. As the pD is increased and the carboxylic acid group is titrated, a binding region with a high affinity for the imidazolium ring is created. The dipositive form of histamine binds site specfically, with its imidazolium group in this binding site and its ammonium group hydrogen-bonded to an I ring carboxylate group. As the pD is increased further, the imidazolium group of free histamine in equilibrium with bound histamine is titrated, which results in dissociation of the heparin–histamine complex.

Perhaps the most surprising result of the present study is the finding of a binding site with a high affinity for the imidazolium ring. This is of particular interest in view of the fact that binding of heparin by a histidine-rich glycoprotein found in platelets, plasma, and serum neutralizes the anticoagulant activity of heparin.[4] We are investigating the interaction of other biological molecules, including histidine-containing peptides, with heparin and related polysaccharides. We also are investigating the binding

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(40) Based on a molecular weight for heparin of 12000, 1 mol of heparin will bind 21.5 mol of histamine (Kobayaski, Y. *Arch. Biochem. Biophys.* **1962**, *96*, 20–27). Using a disaccharide molecular weight of 614, calculated by assuming 3.5 anionic charges per disaccharide unit and that the variation among disaccharide units is in the number of $-OSO_3-$ groups, this corresponds to 21.5 mol of histamine per 19.5 disaccharide units.

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of histamine by heparin derivatives and other glycosaminoglycans to determine the minimum requirements of the imidazolium binding site.

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Registry No. EDA, 107-15-3; PDA, 109-76-2; BDA, 110-60-1; (IdoA-2S)-(1→4)-(GlcNSO$_3$-6S), 76149-63-8; heparin, 9005-49-6; histamine, 51-45-6; imidazole, 288-32-4; methylamine, 74-89-5; *N*-acetylhistamine, 673-49-4.

Spin and Reaction Dynamics in Flexible Polymethylene Biradicals As Studied by EPR, NMR, and Optical Spectroscopy and Magnetic Field Effects. Measurements and Mechanisms of Scalar Electron Spin-Spin Coupling

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Abstract: A series of spectroscopic measurements on flexible polymethylene biradicals in liquid solution is reported. Time resolved electron paramagnetic resonance, chemically induced nuclear spin polarization, transient optical absorption, and external magnetic field effects on lifetimes are used to unravel the kinetics of two types of biradical, acyl-alkyl and bisalkyl, obtained from the photoinduced Norrish type I α-cleavage reaction of permethylated cycloalkanones. A kinetic picture for the biradical exit channels is presented which includes rate constants for spin-orbit coupling, decarbonylation, spin relaxation, and end-to-end contact of the chains. The relative merit of each of the various experimental techniques is discussed, and the close relationship between spin and chain dynamics in the biradical kinetics is clearly illustrated. Direct and indirect measurements of the singlet-triplet splitting (electronic spin-spin coupling) are made and compared for different chain lengths. The mechanism of the coupling as well as its temperature dependence is discussed in terms of through-bond and through-space models. The acyl-alkyl biradicals are shown to have kinetics dominated by spin-orbit coupling, whereas the bisalkyl biradical spectra decay on a slower time scale via spin relaxation. Optical measurement of the decarbonylation rate of the pivaloyl monoradical is also reported.

Introduction

There has been a long standing interest in the chemistry of polymethylene biradicals in these laboratories ever since the first observation of CIDNP upon α-cleavage of cyclic ketones.[1] The recent development of time-resolved electron paramagnetic resonance (EPR) spectroscopy allowing the observation of such biradicals directly at room temperature in liquid solution, under conditions of maximum reactivity, has made it possible to gain new insight on how the reaction dynamics are governed by magnetic parameters.[2] In this paper we wish to present the results of studies on two series of biradicals obtained by EPR, NMR, transient optical absorption, and magnetic field effects on reaction rates. From these studies has emerged a unified picture of the kinetics, plus a better understanding of the relationship between spin and chain dynamics in flexible biradicals.

Before presenting the details of the present study, it will be useful to summarize a few of the previous investigations of biradical kinetics from this and other laboratories. The earliest reported experiments on spectroscopic properties of polymethylene type biradical reactions are the CIDNP investigations of Closs and Doubleday.[1] By using Norrish type I photochemical cleavage of cyclic ketones, they generated acyl-alkyl biradicals and observed the CIDNP spectra of their reaction products. Magnetic field dependence of those signals gave information on the exchange interaction in the biradicals when interpreted with theoretical models. The most complete model used to interpret these data was developed by de Kanter et al.,[3] yielding the parameters for the exponential distance dependence of the exchange coupling constant with which much subsequent work has been explained. Doubleday and Turro with their collaborators[4] used α- and α'-phenylated cyclic ketones for the purpose of providing an optical chromophore, thus making it possible to study the biradicals directly using transient absorption to obtain kinetic data. Development of submicrosecond time resolved CIDNP experiments by Closs, Miller, and Redwine[5] yielded additional kinetic information, particularly on nuclear spin dependent chemical reaction rates. Johnston and Scaiano have recently published a comprehensive review of optical studies on short biradicals (1,6 and shorter), many of them performed in their own laboratories, showing the broad spectrum of lifetimes these intermediates can cover.[6]

All these experiments have in common that the biradicals are generated from a triplet excited ketone and therefore are born with triplet spin functions. The final products of the reactions

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