The Relationship between Counterion Activity Coefficients and the Anticoagulant Activity of Heparin

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ABSTRACT: A series of sodium heparinate preparations have been characterised in terms of their ionogenic groups, utilizing a fluorescence titration technique. Single ion activity coefficients of the counterion were determined in pure polyelectrolyte solutions and polyelectrolyte/NaCl solutions. The results are discussed in relation to the anticoagulant activity of each heparin preparation. There appears to be a correlation between the linear charge parameter of the polyanion, and anticoagulant activity.

Heparin is a naturally occurring mucopolysaccharide of molecular weight 5000–50 000. It is initially synthesized as an alternating copolymer of *N*-acetylglucosamine and glucuronic acid[1] but in subsequent pathways glucosamine residues are N-deacetylated and N-sulfated; glucuronic acid residues epimerise to iduronic acid which together with glucosamine are ester sulfated.[2]

The anticoagulant activity of heparin has been investigated by several researchers in order to correlate the biological role with polydispersity in molecular size,[3] variation in the ratio of glucuronic to iduronic acid,[4] alterations in the amount of ester and N-sulfation,[5] and differing degree of N-acetylation.[6] Although these chemical and physical differences can affect the anticoagulant activity, at present none of these criteria have been shown to be paramount, and for instance molecular weight dependence of anticoagulant activity has given rise to a number of conflicting reports.[3,7,8] This implies that the anticoagulant activity of heparin might be related to a variety of chemical and physical factors.[9]

In this present investigation, preparations of sodium salts of heparin were examined in an attempt to relate the cationic binding strength both in the pure polyelectrolyte system and also with added sodium chloride to the anticoagulant activity of heparin. Stivala et al.[10] have already proposed that a correlation exists between Cu(II) binding and the anticoagulant activity of heparin. Also it has been noted that a relationship exists between the counterion salt form of a heparin preparation and its efficacy as a prophylactic drug for deep venous thrombosis and local haematoma treatment.[11-13] There is, therefore, a necessity to provide a better understanding of the parameters governing anticoagulant activity.

It is generally known[14] that polyelectrolytes characterised by the dimensionless linear charge parameter, ξ, where $\xi > \xi_{critical}$, have a fraction of condensed counterions, $(1 - |z_i|\xi^{-1})$

$$\xi = \frac{e^2}{\epsilon k T b} \tag{1}$$

where e is the protonic charge, ϵ is the bulk dielectric constant of the solvent, k is Boltzmann's constant, T is the absolute temperature, and b is the average distance between charged groups on the polyelectrolyte chain; $\xi_{critical}$ is given by $|z_i z_p|^{-1}$, where z_i is the charge carried by the counterion and z_p is the charge carried by the charged site of the polyelectrolyte.

The Manning model for the infinite line charge gives rise to limiting laws for thermodynamic parameters, and these laws received extensive experimental testing, and have been found to give good agreement between theory and experiment[15-17] for single counterion species, although deviations have been found[18] for mixed counterion systems. We have used the Manning model throughout as we are here dealing with a single counterion system, although under certain circumstances the cluster model developed recently by Iwasa[18] might prove more appropriate for some workers, as the theory is not thoroughly substantiated. The Manning model involves approximating the polyelectrolye to a rigid rodlike structure and ionic polysaccharides fulfil this requirement to an acceptable degree.

The experimental data treated with Manning's theory are discussed in relation to the chemical composition and biological function of the heparin preparations.

Experimental Section

Purified heparin fractions of different anticoagulant activities (extracted from hog mucosa), 165, 157, 144, 68, and 22 units/mg (hereafter denoted as 165, 157, etc.), were supplied by Abbott Laboratories, North Chicago, Ill. (165, 157, 68, 22) and Weddel Pharmaceuticals Ltd, London (144). Equivalent weights of the polyanions were obtained by spectrofluorimetric titration with the cationic dye Acridine Orange.[19] Acridine Orange was excited on the short wavelength of the monomer absorption maximum, at 400 nm. Fluorescence is viewed at 540 nm, rather than at the maximum intensity of 530 nm, so as to minimize inner filter effects from self-absorption of the fluorescence. Heparin ($\sim 5 \times 10^{-5}$ M) was added in 0.5-mL aliquots to 20 mL of Acridine Orange (1×10^{-5} M) and the change in the relative fluorescent intensity of the dye monomer at increasing concentrations of heparin was measured using a Perkin-Elmer MPF-43A. Titrations were carried out at pH 7 to find the equivalent weight of the polyanions using a 1:1 equivalence of polyanion site to dye as the end point, and at pH 3 in order to calculate the sulfate/carboxylate (SO_3^-/CO_2^-) ratio of the heparin samples. Both the heparin and Acridine Orange solutions were adjusted to pH 3 by the addition of 0.1 M hydrochloric acid. Using the experimental values of the SO_3^-/CO_2^- ratios, the idealized repeating structure of heparin in each case was adjusted to give a SO_3^-/CO_2^- ratio corresponding to the experimentally determined values, based on the *N*-sulfate being the most labile sulfate group. This, together with the total acid group content, allowed a computation to be performed to obtain a theoretical equivalent weight which correlated reasonably well (in all cases the difference is <3%) with the experimental equivalent weight obtained by the fluorescence titration method (Table I). With this information we could obtain a reliable degree of substitution and hence get a value for the linear charge parameter, ξ. The value of ξ was determined by making molecular models of the sugar units in their most extended form, from which we can calculate a value of 20.4 Å for the length of a tetrasaccharide unit. This compares with a value of 21.1 Å, from X-ray scattering measurements, for the persistence length of heparin tetrasaccharide calculated by Stivala et al.[20] All these data are presented in Table I. Activities of the sodium counterion were found using the previously described[16] ion selective electrode technique and the single ion activity coefficients obtained from this and a knowledge of the molal concentration of the counterion. The lowest counterion concentrations investigated were 6.25×10^{-4} m in sodium chloride and 2.5×10^{-3} m in sodium heparinate. The experimental equivalent weights from Table I were used in preparing heparin solutions of the appropriate molality. Analar grade sodium chloride and distilled water were used in all experiments. Moisture contents were determined by drying ov-

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Table I
Chemical Composition and Linear Charge Parameters for Heparin Samples

a	equiv wt of sodium heparinates: exptl	equiv wt of sodium heparinates: calcd	SO_3^-/CO_2^-	ξ [b]
22	223	217	2.15	1.66
68	207	211	2.33	1.75
144	186	186	2.05	2.10
157	185	182	2.37	2.33
165	178	177	2.41	2.45

[a] Heparin anticoagulant activity in units/mg. [b] Linear charge parameters.

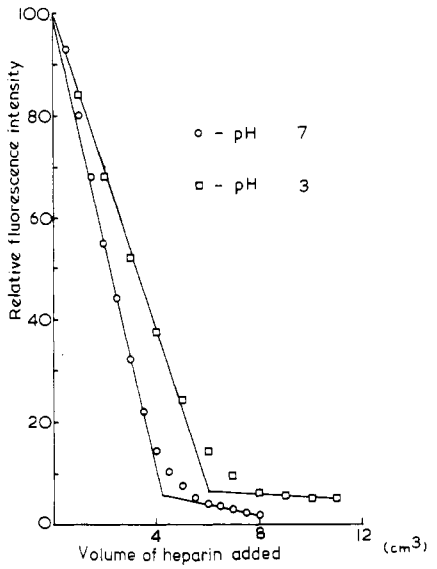

Figure 1. The spectrofluorimetric titation of heparin (165 units/mg) solution ($\sim 5 \times 10^{-5}$ M) with Acridine Orange (1×10^{-5} M). Graph of the decrease in dye fluorescence intensity with increasing heparin concentration: (a) pH 7.0 (b) pH 3.0.

ernight in a vacuum oven at 40 °C.

Results

Figure 1 shows that increasing amounts of heparin decrease the fluorescence intensity of the monomeric dye Acridine Orange at 540 nm. As polyanion concentration increases to a 1:1 equivalence of anionic sites to dye, fluorescence intensity stems principally from the bound dye, giving an emission with λ_{max} at 630 nm.[19] A plot of decrease in monomer absorption against concentration of heparin indicates the number of sites on the polysaccharide, the end point being determined by locating the point of intersection of the extrapolations of the linear sections of the curve. At pH 7, both the carboxylate and sulfate groups are ionized, whereas at pH 3 the carboxylate groups are protonated and unavailable for interaction with Acridine Orange. The end points of the titrations at pH 7 were used to determine the equivalent weight of the various heparin samples and titrations at pH 7 and 3 used to determine the SO_3^-/CO_2^- ratio (Table I). Table I shows that there is no direct correlation between the SO_3^-/CO_2^- ratio and the anticoagulant activity of the heparin samples examined.

Figure 2 shows the concentration dependence of the single ion activity coefficient of the sodium ion in the heparins of different anticoagulant activity. While the concentration dependence was not particularly pronounced, the results are unusual in that a decrease of activity coefficient with dilution was observed rather than an increase with dilution. Figure 3 shows the single ion

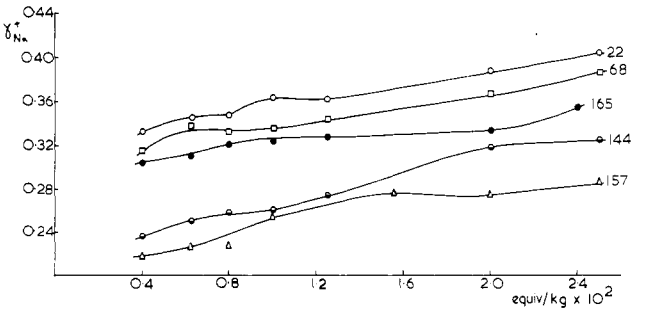

Figure 2. Activity coefficients of sodium counterions γ_{Na^+} against concentration for sodium salt of heparins.

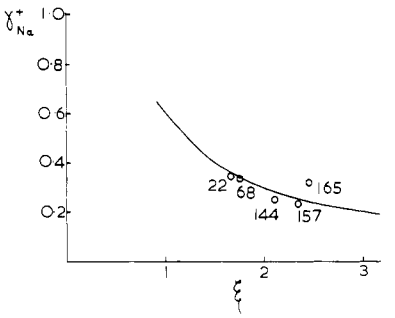

Figure 3. Activity coefficients γ_{Na^+} of sodium counterion in the most dilute solution against the linear charge parameter.

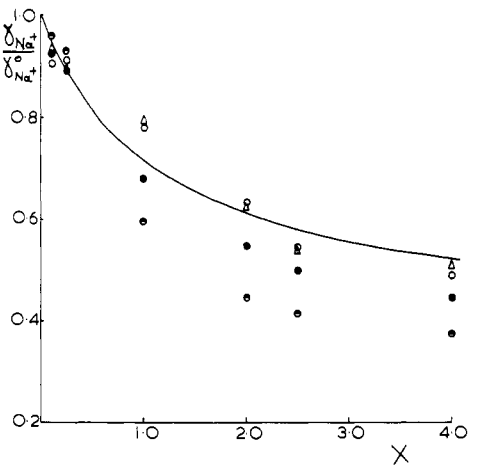

Figure 4. Activity coefficients γ_{Na^+} of the common cation in the sodium heparinate 22/sodium chloride mixture, corrected for small ion–small ion interactions. Comparison with theoretical curve according to eq 3. Sodium heparinate: (△) 1×10^{-2} m, (○) 8×10^{-3} m, (●) 5×10^{-3} m, (◓) 2.5×10^{-3} m.

activity coefficient of the sodium counterion of each heparinate in the most dilute solution in which measurements could be made, and this is approximated to the limiting value for γ_{Na^+}, given by eq 2. It can be seen that the

$$\gamma_{Na^+} = e^{-1/2}/|z|\xi \qquad (2)$$

agreement with the theoretical limiting value of γ_{Na^+} denoted by the solid line is quite reasonable for heparins 22, 68, 144, and 157, with a greater deviation being observed for heparin 165.

In Figures 4–8, the single ion activity coefficients of the sodium counterions are presented for sodium heparinate/sodium chloride mixtures in the form of $\gamma_{Na^+}/\gamma^0_{Na^+}$. γ_{Na^+} is the single ion activity of the sodium ion in the presence of the heparinate polyanion, and $\gamma^0_{Na^+}$ is the single ion activity coefficient value of the sodium ion in the absence of the polyanion, where $\gamma^0_{Na^+}$ is estimated for the appropriate sodium ion concentration using the Debye–Hückel equation in its extended form, with the ap-

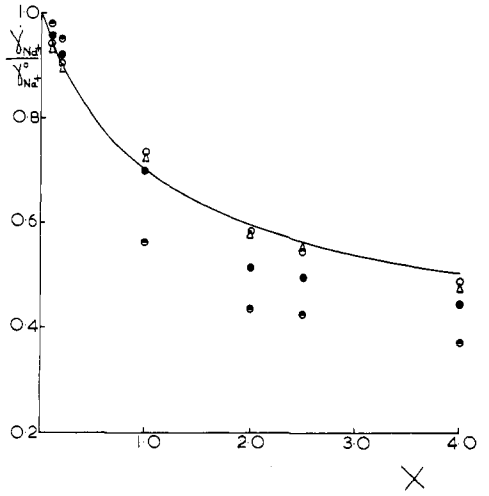

Figure 5. Activity coefficients γ_{Na^+} of the common cation in the *sodium heparinate 68/sodium chloride mixture, corrected for small* ion–small ion interactions. Comparison with theoretical curve according to eq 3. Sodium heparinate: (△) 1×10^{-2} m, (○) 8×10^{-3} m, (●) 5×10^{-3} m, (◓) 2.5×10^{-3} m.

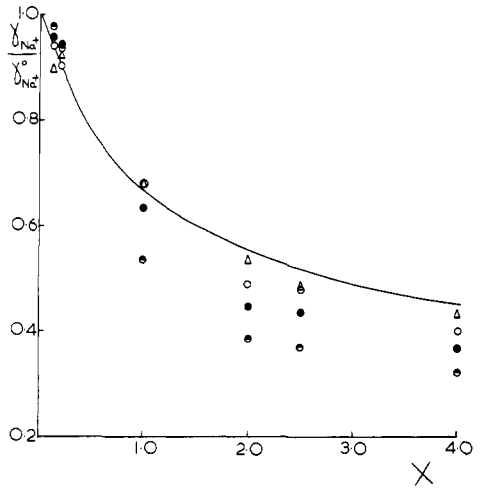

Figure 6. Activity coefficients γ_{Na^+} of the common cation in the sodium heparinate 144/sodium chloride mixture, corrected for small ion–small ion interactions. Comparison with the theoretical *curve according to eq 3. Sodium heparinate:* (△) 1×10^{-2} m. (○) 8×10^{-3} m, (●) 5×10^{-3} m, (◓) 2.5×10^{-3} m.

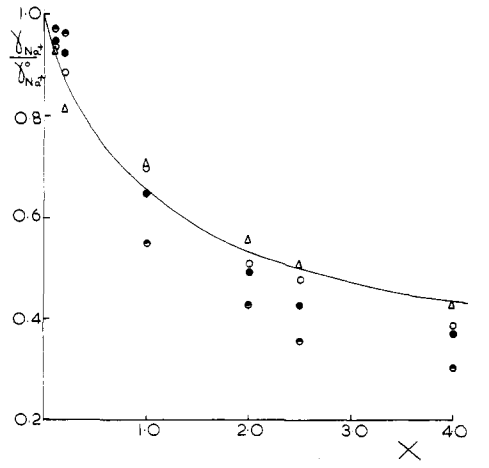

Figure 7. Activity coefficients γ_{Na^+} of the common cation in the sodium heparinate 157/sodium chloride mixture, corrected for small ion–small ion interactions. Comparison with the theoretical curve according to eq 3. Sodium heparinate: (△) 1×10^{-2} m, (○) 8×10^{-3} m, (●) 5×10^{-3} m, (◓) 2.5×10^{-3} m.

propriate ion size parameter.[16] This accounts for small ion–small ion interactions, which is not allowed for in the original Manning model.[21] γ_{Na^+} values were determined in aqueous solutions of sodium chloride in the concentration range 8×10^{-2} to 6.25×10^{-4} m containing 1×10^{-2}, 8×10^{-3}, 5×10^{-3}, and 2.5×10^{-3} m sodium heparinate. The results for all sodium heparinate/sodium chloride mixtures showed the same trend, a decrease in $\gamma_{Na^+}/\gamma^0_{Na^+}$ as a function of X, X being the ratio of the number of equivalents of polyelectrolyte to the number of equivalents of simple electrolyte within unit volume. For values of $X < 1$, the spread of $\gamma_{Na^+}/\gamma^0_{Na^+}$ values for a given value of X is small compared to the spread of values for $X > 1$. For a given value of X, $\gamma_{Na^+}/\gamma^0_{Na^+}$ appears, generally, to increase in value with decrease in salt concentration for $X < 1$, and to decrease with decrease in salt concentration for $X > 1$. Using the infinite line charge model of Manning, the theoretical equation for the single ion activity coefficients is, for all the sodium heparinate/sodium chloride mixtures, given by eq 3, since ξ is always greater

$$\gamma_{Na^+} = (\xi^{-1}X + 1)(X + 1)^{-1} \exp\left[\frac{(-\frac{1}{2}\xi^{-1}X)}{(\xi^{-1}X + 2)}\right] \quad \text{for } \xi > 1 \quad (3)$$

than 1. The theoretical value of γ_{Na^+} is plotted for the corresponding value of ξ given in Table I, as a function of X for each sodium heparinate, and this corresponds to the solid lines in Figure 4–8. The agreement between the experimental points and the theoretical curve is quite reasonable for low values of X. At higher values of X, the agreement is only good for a higher concentration of added simple electrolyte.

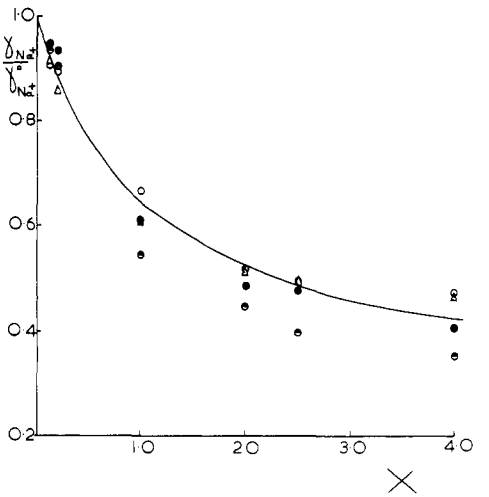

Figure 8. Activity coefficients γ_{Na^+} of the common cation in the sodium heparinate 165/sodium chloride mixture, corrected for small ion–small ion interactions. Comparison with the theoretical curve according to eq 3. Sodium heparinate: (△) 1×10^{-2} m, (○) 8×10^{-3} m, (●) 5×10^{-3} m, (◓) 2.5×10^{-3} m.

Discussion

The purpose of this present study was primarily to investigate the sodium counterion binding properties of a series of heparins, since the heparins have received relatively little detailed attention in this respect.[22,23] We also hoped to see if there was any relationship between their sodium ion binding properties measured in terms of the single ion activity coefficient, and the anticoagulant action of some commercially available heparin preparations. The anticoagulant action of heparin is essentially derived from its ability to bind to antithrombin III[24] and other serine proteases. There have been numerous attempts to directly relate the chemical and physical differences of heparin samples to their biological action.[25]

The sodium counterion activity coefficient is a parameter which is a much more homogeneous property of each heparin and it should also be noted that it is the sodium form of heparin which is extensively administered as an anticoagulant. In vivo, calcium ions will replace site bound (or condensed) sodium ions.[26] *However*, the type of counterion present has been shown not to affect the anticoagulant activity of the heparin,[27] while it should be recognized that specifically calcium ions are a necessary constituent of the blood clotting reaction.

It is observed with fully sulfated polyanions such as the carrageenans[16] and dextran sulfates[28] and the fully carboxylated polyanions such as the alginates[29] that the single-ion activity coefficients of the monovalent counterions increase with dilution in salt-free solution. In contrast, with the heparins the single-ion activity coefficient in salt-free solution decreases with dilution. Other workers have observed, however, that the single-ion activity coefficients of polyelectrolytes increase with concentration to give a value in excess of the limiting value according to Manning's theory.[30] This deviation is ascribed mainly to a breakdown of the rodlike geometry of the polyelectrolyte at higher concentrations, in agreement with Yuan's observations that heparin in solution exists in a contracted form.[31] Yuan et al.[23] found that for unfractionated sodium heparinate in water, in the concentration range $1\text{–}10 \times 10^{-4}$ mol/L, an order of magnitude lower than our concentrations, that there was no concentration dependence of counterion single-ion activity coefficient. In Figure 3, the quite reasonable agreement between the limiting value of γ_{Na^+}, taken in the most dilute solution in which measurements could be made, and Manning's predicted limiting values, given by the solid line, is probably purely fortuitous since Manning's theory does not take account of specific counterion effects which have been reported on numerous occasions.[16,29] From the data obtained by the fluorimetric titration technique, the values of degrees of substitution, SO_3^-/CO_2^-, and ξ in Table I could be obtained for each heparin sample. It is seen that there is a direct relationship between the calculated linear charge parameter, a nonadjustable parameter, and the anticoagulant activity of each sample, with a higher linear charge parameter corresponding to a higher anticoagulant activity. The linear charge parameter, according to Manning's theory, is determined for a polyanion in its most extended form. Yuan observed that heparin in solution exists in a less fully extended form. Thus the agreement between the theoretical value of γ_{Na^+} according to eq 2 for the calculated value of ξ and the observed value of γ_{Na^+} in the most dilute solution measured is very good (Figure 3), apart from the value for heparin 165. It can be seen that heparins with a greater fraction $(1 - \xi^{-1})$ of condensed counterions have a greater anticoagulant activity.

It can also be seen from Table I that in this study no correlation exists between the SO_3^-/CO_2^- ratio and anticoagulant activity. However, the anticoagulant activity of heparin preparations has been shown to decrease with loss of sulfate.[32] For the present data it is suggested that other chemical and physical differences between the heparin samples may account for this result. Thus, the more important feature determining biological activity of a heparin appears to be the function of condensed counterions, rather than of the ionogenic groups involved. The greater sodium ion sequestering effect of a heparin with a higher linear charge parameter may well leave more sites on antithrombin III for a heparin molecule to interact with antithrombin III, resulting in a higher anticoagulant activity.

In Figures 4–8 the agreement of the $\gamma_{Na^+}/\gamma^0_{Na^+}$ values with the solid line for a given value of X, according to eq 3, is good for values of $X > 1$, when the heparin and sodium chloride concentration are maximized. The deviations at lower heparin and sodium chloride concentrations are negative deviations of similar type to those observed for sodium dextran sulfate/sodium chloride mixtures.[33] On the whole, for the sodium heparin/sodium chloride mixtures, when the polyanion is in excess, i.e., $X > 1$, the activity coefficient expressed as $\gamma_{Na^+}/\gamma^0_{Na^+}$ is greatest for a given value of X, when the concentration of heparin and sodium chloride is greatest and for $X < 1$; $\gamma_{Na^+}/\gamma^0_{Na^+}$ is greatest when the concentration of heparin and sodium chloride is smallest.

We conclude that there is an obvious correlation between the linear charge parameter of a sodium heparinate preparation (and hence its ion binding properties) and the anticoagulant properties it manifests in line with the previous findings of Stivala.[10] This lends weight to the thesis that it is essentially the ion binding properties of heparins in relation to antithrombin III that is a prime controlling factor in blood coagulation, and this is further shown to be an important parameter by the recent work of Herwats et al.[34] when it was shown that a natural polypeptide (factor 4), an antiheparin, displaces cations from anionic sites on the heparin molecules when inactivation occurs.

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Some Photophysical Properties of Five New Carbazole-Containing Methacrylate Polymers

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ABSTRACT: Five new carbazole-containing polymers have been synthesized. With poly(*N*-vinylcarbazole) these form two series in which (a) the nitrogen atom of the chromophore is attached to the polymer backbone with an increasingly flexible linking moiety, PNVK, I, -II, -III, and (b) a series in which the backbone is attached to the 9-, 2- and 3-ring positions, I, IV, V. The molecular weights of the polymers have been characterized by membrane osmometry and gel permeation chromatography. These polymers are susceptible to photochemical degradation which causes both scission of the chain backbone and diminution of carbazole chromophore fluorescence. Sensitive viscosity measurements made on samples undergoing this main-chain scission have been used to obtain the intrinsic viscosity–molecular weight K, α values of the resulting fragments. Comparison of the absorption and emission spectra shows that appreciable excimer emission occurs only in the case of poly(*N*-vinylcarbazole) (PNVK) and poly[2-(9-ethyl)carbazolylmethyl methacrylate] (IV). Excimer emission from the latter is weakest in polar fluid solvents and strongest in a rigid glassy matrix. Luminescence decay measurements in the presence and absence of anthracene quencher suggest that down-chain energy migration is virtually absent in the sterically unhindered polymers I, II, III, and V, and this is confirmed by steady state analysis of collisional quenching. Collisional quenching of monomer excitation from IV indicates that the excitation is effectively immobilized in the region of not more than three neighboring chromophores. These results show that the flexibility of the linking group does not assist the adoption of the parallel overlap interchromophore geometry necessary (to different extents) for both excimer formation and resonance energy migration. However, geometrical constraints imposed by chain linking to the chromophore 2 position (polymer IV) do favor (relative to attachments at the 9- and 3-ring position) the adoption of a ground state interchromophore geometry permitting excimer formation with relatively minor conformational readjustment or monomer excitation energy migration.

The importance of electronic energy migration and transfer, and of excimer and exciplex formation, is now widely recognized in a variety of photochemical and photophysical phenomena. Polymeric materials in which neighboring chromophores are brought into proximity by the constraints of the chain backbone have considerable potential for exhibiting unique energy transfer characteristics. Thus previous studies have shown that it is possible to measure, quantitatively, down-chain energy migration coefficients in a variety of polymers,[1,2] that selected chromophores may be attached to the chain backbone with different geometries so giving different photophysical properties,[3,4] and that energy transfer and excimer formation are affected by external control of polymer chain conformations.[5,6]

The considerable technical and academic interest in the photoelectric properties of poly(*N*-vinylcarbazole) (PNVK) has led to many detailed studies of the excimer formation and singlet energy transfer properties of this polymer.[1,7-11] These studies have shown that in this polymer excimer formation (and therefore probably also energy migration) is affected by stereochemical configuration and by the adoption of a helical conformation.

The objective of this work was to study further the photoproperties of polymers containing the carbazole chromophore, and to determine how the properties are affected by the geometrical nature of the attachment of the chromophore to the chain backbone. To this end two series of polymers have been synthesized in which (a) the chain backbone is attached to the N atom at the 9 position by progressively larger and more flexible linking units and (b) the chain backbone is attached (via a methyl ester moiety) to the 9-, 2-, and 3-ring positions.

Experimental Section

1. Synthesis of Monomers. Five new carbazole-containing monomers (Ia–Va) were synthesized. Details of these reactions, and analytical data characterizing the novel reaction intermediates, will be published elsewhere. The syntheses produced good yields of crystalline monomers which were finally purified by column chromatography.

2. Synthesis and Characterization of Polymers (I–V) from Monomers (Ia–Va). The carbazole-containing poly(meth-

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