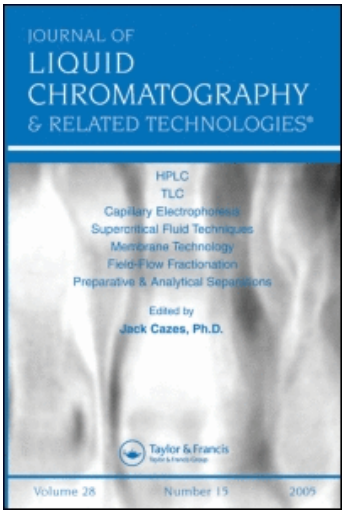

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SOLUTION PROPERTIES OF POLYELECTROLYTES. VI. SECONDARY EFFECTS IN AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY

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ABSTRACT

An independent analysis of different operational variables in aqueous size exclusion chromatography of polyelectrolytes has been carried out using a silica-based support. The effect of polyion concentration, pH and ionic strength on sodium polystyrenesulfonate calibration plots has been investigated. Finally, a novel semi-empirical model has been developed from thermodynamic considerations which relates the support effective pore volume to the polyelectrolyte molecular weight and qualitatively describes secondary effects.

INTRODUCTION

Aqueous size-exclusion chromatography (ASEC) is currently a technique commonly used to characterize water-soluble ionic and nonionic polymers as well as biopolymers (1). Pure or "ideal" ASEC requires that the chromatographic

separation is governed exclusively according to the hydrodynamic properties of the macromolecule. However, in the case of strong polyelectrolytes this requirement is difficult to be met due to the appearance of secondary effects such as ion-exclusion and hydrophobic interaction which cause shifts in elution volumes. At present, the available hydrophilic gels exhibit surface residual charges which interact with polyion charges preventing the elution mechanism from being "ideal". Although these interactions can be attenuated, their complete suppression is practically impossible (2). Abundant experimental evidence from numerous reports on secondary effects in ASEC demonstrates that these effects occur for organic as well as inorganic supports. Thus, Dubin (3,4) has made an attempt to qualitatively evaluate the influence of the substrate surface charge on the elution of polyelectrolytes. Potschka (5) has evidenced that the effective radii of proteins increase in reverse proportion to the square root of the ionic strength, and Mori (6) has proposed a procedure to elucidate the retention behavior of ionic polymers. Also, a thorough study of the behavior of polyanions and polycations in ASEC has been carried out in our lab in experimental conditions similar to those employed by other authors, including salt-free, salt-containing and buffered eluents, using both silica-based and cross-linked organic supports (7-10). In this regard, injected sample concentration is one of the operational variables thoroughly studied by us and rarely analyzed in-depth by other groups.

In the present contribution the elution behavior of sodium poly(styrenesulfonate) (NaPSS) and linear polysaccharide (dextran) is investigated using a silica-based support. Different experimental parameters such as polyion concentration, c_p, eluent ionic strength, c_s, and eluent pH have served to obtain a set of calibration curves. The divergence between the curves for

NaPSS and dextran has been considered in order to analyze the secondary effects between the polyion and the substrate. The determination of the intrinsic viscosity, $[\eta]$, for NaPSS at low ionic strength is particularly problematic because it is not possible to extrapolate the reduced viscosity at zero concentration (11). An equation to predict the intrinsic viscosity of a polyion at finite c_p and c_s has been recently developed (9,10). On the other hand, an attempt has been recently made to quantify the secondary effects in ASEC of polyelectrolytes, by introducing the concept of a molecular weight-dependent effective pore volume, V'_p, which has been justified on the basis of an electrostatic potential between macromolecule and substrate surface charges. In the present paper, assuming the existence of V'_p and taking into account a thermodynamic formalism recently developed by us to predict secondary effects in SEC of uncharged polymers (12,13), a semi-empirical equation is proposed to relate V'_p to the polyion molecular weight.

EXPERIMENTAL

Chemicals and reagents

NaPSS samples were dialyzed fractions of commercial standards purchased from Pressure Chemical Co. (Pittsburgh, PA, USA), of molecular weights, M, 1600; 4000; 16000; 31000; 88000; 177000 and 354000 g/mol, with polydispersity lower than 1.1. Dextran standards were obtained from Pharmacia Fine Chemical (Uppsala, Sweden), of molecular weights 10000; 17700; 40000; 83000 and 170000 g/mol.

All reagents used in the preparation of buffers were analytical grade from Merck (Darmstadt, F.R.G.). HPLC grade water (Merck) was daily tested by

means of a CRISON conductimeter, model 522, the mean conductivity value being $(1.9\pm0.5)\times10^{-6}\ \Omega^{-1}cm^{-1}$.

Viscosity

Viscosity measurements were performed with a calibrated Ubbelohde-type viscometer, thermostated at 25.00 ± 0.01 °C. Flow times were determined to 0.01 s.; the flow time of the pure solvent was always higher than 110 s. Kinetic energy corrections were included in the calculations of specific viscosities. Solutions of NaPSS were prepared by weight and equilibrated at 25.00 °C for several days prior to being placed into the viscometer. The initial concentration was 0.4-0.6 g/dL in all cases. The viscometric equation used for dextrans was in all instances $[\eta] = 97.8\times10^{-3}M^{0.50}$ (14), because as previously indicated, the influence of salt on the viscosity of non-ionic polymers such as those mentioned above can be neglected (15).

pH measurements

pH measurements were performed with a Radiometer-pHmeter 29 (Copenhagen, NV). The dependence of solution pH on NaPSS concentration in the range from 0.25 to 10 g/L was determined in three different buffered solvents. Measured pH values during the titration were stable, with no significant drift.

Chromatographic measurements

A Waters liquid chromatograph (Waters Chromatography Division, Millipore, Milford, MA) equipped with an M-45 solvent delivery system, a U6K universal injector and an R-410 refractive index detector coupled to an SP 4290

automatic recorder (Spectra-Physics, San José, CA), was used. A Waters Protein I-250 column with a separation range from 10 to 500 kD for globular proteins and from 2 to 150 kD for denatured proteins (16) was used.

The eluents employed were in all cases buffers at pH values of 7.0 and 5.9 (phosphate) and 4.0 (acetate), degassed and filtered through regenerated cellulose 0.45-μm pore diameter filters from Micro Filtration Systems (Dublin, CA). The column was equilibrated for at least 12 hours prior to starting any experiment. Polymer solutions were always prepared with the corresponding mobile phase as solvent. The flow rate was 1.0 mL/min. at a pressure of 30 bar. Each sample was injected in triplicate and the average value taken in each case. Injected volume was 100 μL in all cases and NaPSS concentrations assayed were 10.0; 8.0; 3.0; 1.0; 0.5 and 0.1 g/L. The dextrans calibration curve was obtained by extrapolation at infinite dilution of at least three concentrations and resulted to be independent from pH and ionic strength in the range of values studied.

RESULTS AND DISCUSSION

Viscosity

The intrinsic viscosity of polyelectrolytes is a magnitude necessary to perform calibration curves, log $M[\eta]$ vs. retention volume, in ASEC. Most researchers in this field use the phenomenological equation proposed by Fuoss in the 1940s (17). However, in certain conditions such as weakly acidic solvents or low-salt content solutions (as is the case in the present work), the reduced viscosity exhibits a pronounced maximum ocurring at a concentration which is independent from molecular weight and polydispersity (18). In this case, the

Fuoss law is not appropriate to predict the viscosity of a dilute polyelectrolyte solution, and hence, its application and further extrapolation to zero concentration yields a great uncertainty on the intrinsic viscosity value. Recent theories postulate the dependence of viscosity on polyelectrolyte concentration, molecular weight, linear charge density and solution ionic strength (19-22). In a preceeding paper (9), a new equation has been proposed to predict the intrinsic viscosity of NaPSS at finite polyion concentration and low salt concentration:

$$[\eta]_{p,c_p,c_s} = \frac{D_p + A_p\beta^{-1} + 0.5A_p\beta^{-2}\left[3(\beta-1)(c_p+c_s)^{-1} - 4B_p^{1/2}\right]c_p}{1+(D_p+A_p\beta^{-1})c_p} \qquad [1]$$

where $\beta = 1+\alpha^2(c_p+c_s)^{3/2}$ and $\alpha = (B_p^{1/2}c_p + B_s^{1/2}c_s)(c_p+c_s)^{-3/2}$

A_p, D_p and A_s, D_s correspond to viscometric constants for the polyion and the single electrolyte present in the solution, respectively. More details about this equation and its applications in ASEC of polyanions and polycations have been previously reported (9,10).

In order to check eqn.[1], experimental and predicted intrinsic viscosity values were compared in different buffer systems. Fig. 1 shows as an example the results for NaPSS of molecular weight 177000 g/mol. It can be seen that a good agreement is generally obtained (see also refs. 9 and 10).

Calibration plots

In order to analyze secondary effects in ASEC, sets of calibration curves $\log M[\eta]_{p,c_p,c_s}$ vs. elution volume were obtained as a function of different

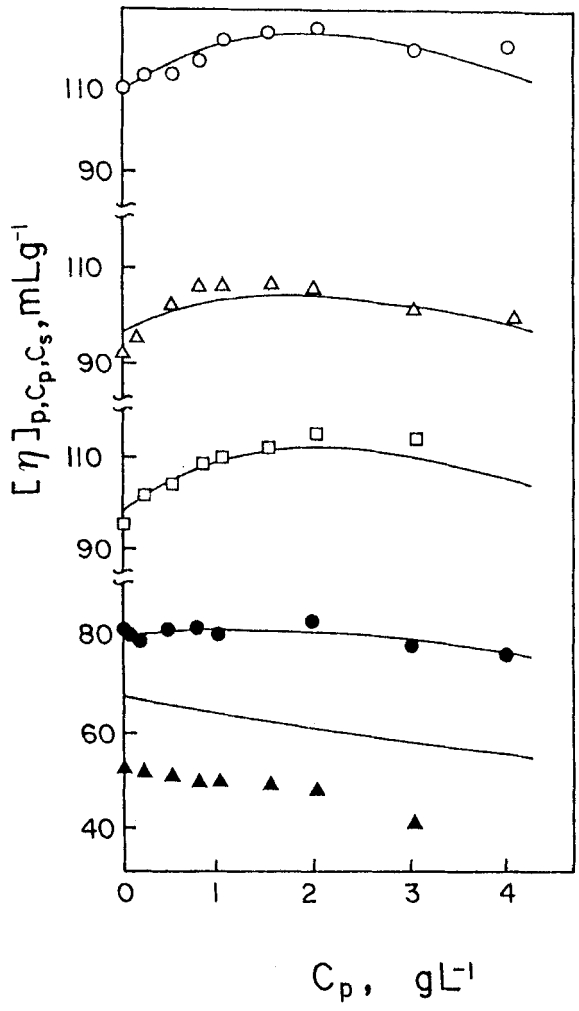

FIGURE 1. Comparison between experimental (symbols) and predicted with eqn.[1] (curves) $[\eta]_{p,c_p,c_s}$ values for NaPSS (177000) as a function of polyion concentration, c_p in: (○) 0.02 M phosphate buffer, pH 7.0; (△) 0.05 M phosphate buffer, pH 5.9; (□) 0.02 M acetate buffer, pH 4.0; (●) 0.05 M acetate buffer, pH 4.0 and (▲) 0.20 M acetate buffer, pH 4.0.

experimental variables, c_p, pH and c_s. The divergence between polyion and dextran calibrations was taken as a criterion to check the individual contribution of each variable to the separation mechanisms. Because the elution volumes for uncharged polymers were independent from pH and c_s, the corresponding calibration curve was considered as a reference system, assuming that elution occurs through a pure SEC mechanism. The influence of c_p for dextrans was not contemplated here because concentration effects for uncharged polymers in aqueous media are similar to those in organic media, as widely reported in the past (23-27).

Effect of polyion concentration

Figs. 2A, 2B and 2C depict the influence of c_p, pH and c_s respectively on the calibration curves for NaPSS. Fig. 2A shows the influence of c_p in the range from 0.1 to 10.0 g/L in phosphate buffer (pH 7.0, c_s=0.02 M) as eluent, purposely selected to enhance the concentration effect. All elution volumes are lower than the corresponding homologous for dextran, which indicates that ion-exclusion takes place. However, this secondary effect diminishes as c_p increases. This decrease could be due simultaneously to both, on the one hand, a diminution in linear charge density weakening the repulsive interactions between NaPSS and residual silanol charges on the support, and on the other, a decrease in molecular size as a result of a shift of the rod↔gaussian coil conformational equilibrium (shrinking effect similar to that caused by an increase in single electrolyte concentration (8,22)).Note that, conversely to the behavior of uncharged polymers (24-26), concentration effects for NaPSS are more pronounced as molecular weight decreases. Polyion concentrations clearly higher than 10 g/L are

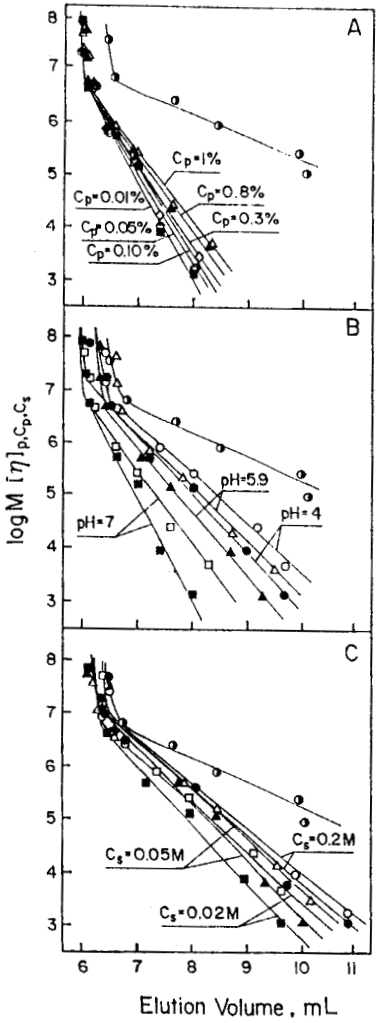

FIGURE 2. Calibration plots, log $M[\eta]_{p,c_p,c_s}$ vs. retention volume, for NaPSS and dextrans (◑) obtained with a Protein I-250 column. (A): Influence of injected sample concentration, c_p, with 0.02 M phosphate buffer, pH 7.0, as eluent. (B): Dependence on eluent pH at 0.02 M ionic strength. Injected sample concentration, c_p: 0.1 (filled symbols) and 10.0 g/L (empty symbols). (C): Influence of eluent ionic strength, c_s, at pH 4.0. Empty and filled symbols correspond to the same c_p values as in case (B).

not recommended because viscous fingering (28) and macromolecular crowding (29) appear distorting the chromatograms and preventing an accurate location of the peak elution volumes. Thus, characterization of NaPSS under these elution conditions (phosphate buffer, pH 7.0, c_s=0.02 M), even at c_p=10.0 g/L, is rather inadequate due to the low separation efficiency obtained.

Effect of pH

In Fig. 2B the influence of pH on the elution of NaPSS is analyzed at a constant c_s=0.02 M. In order not to overcrowd the Figure, the results for only two extreme c_p values corresponding to 0.1 (filled symbols) and 10.0 g/L (empty symbols) have been plotted for each pH assayed. Intermediate c_p values yielded calibration curves (not shown) included between these two limits. All NaPSS calibration curves appear to the left of the dextran reference evidencing again an ion-exclusion effect. The curves at pH 7.0 are farthest from the reference, exhibiting the poorest resolution (especially at low c_p). As a trend, as pH is lowered, calibrations approach the reference, but a certain overlapping occurs so that for pH 5.9 and c_p=10.0 g/L the resolution is slightly better than for pH 4.0 and c_p=0.1 g/L. Finally, with acetate buffer as eluent (pH 4.0, c_s=0.02 M) and for c_p=10.0 g/L the curve is closest to the reference. In the light of these results, an effort is made next to analyze more in-depth the pH-dependent deviations observed.

As concerning the polyelectrolyte, the influence of pH on the extent of dissociation of NaPSS can be assumed to be negligible since the pK_a of sulfonic group (strongly acidic) is always lower than the pH experimentally measured at any c_p and c_s values. Fig. 3 shows the variation of the measured pH of three

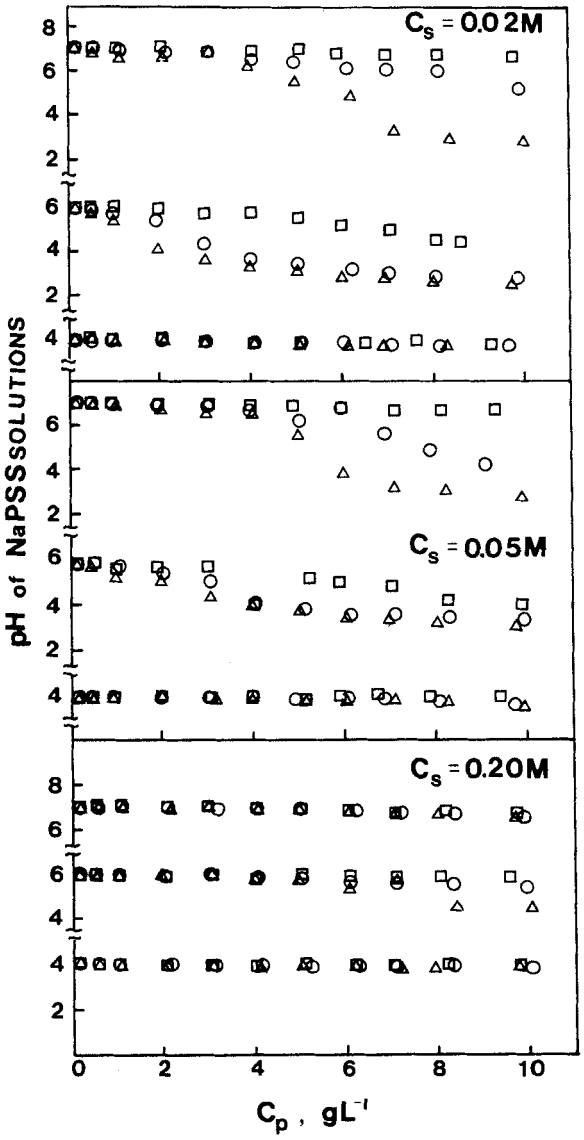

FIGURE 3. Concentration dependence of NaPSS solution pH at indicated ionic strengths, c_s. For each c_s the pH of the pure solvent corresponds from top to bottom to 7.0; 5.9 and 4.0 respectively. NaPSS molecular weight: (□) 31000; (○) 88000 and (△) 177000 g/mol.

different molecular weight NaPSS samples (31, 88 and 177 kD) as a function of c_p, at three ionic strengths of different buffers used as solvents. As can be observed, the influence of c_p on the solution pH with the pH 4.0 buffer is in all cases practically negligible. On the contrary, the combination of low ionic strength (c_s=0.02 M) and high pH (7.0) causes a marked deviation of the solution pH with respect to the buffer (solvent) pH, from c_p~ 5 g/L. Nevertheless, these deviations are not expected to affect the extent of dissociation of the polyion sulfonic groups which will remain dissociated. Moreover, the influence of the pH variation on the intrinsic viscosity can also be discarded because, as has been experimentally evidenced, ionic strength rather than pH is the major factor affecting the hydrodynamic volume (3).

Let us now analyze the influence of pH on the chromatographic support. Assuming a pK_a~6 for the residual silanol groups on the gel surface (30) the equilibrium between protonated and unprotonated forms will be shifted in one direction or another depending on the eluent pH:

$$\equiv SiOH \rightleftharpoons \equiv SiO^- + H^+$$

However, for chromatographic purposes it is important to distinguish between eluent pH and the pH of the injected NaPSS solution. Whereas the first one is constant the second one depends on c_p, this dependence being more pronounced as buffer ionic strength decreases and molecular weight increases (Fig. 3). Changes in the actual injected sample pH with respect to eluent pH could in principle cause some local, transient pH fluctuations which could affect the extent of repulsive interaction between polymer and substrate. These contributions, though small, could add to the general trend observed for the calibrations as a function of pH at different c_p values (see Fig. 2B). Note also that eluent pH

values much lower than 4.0 suggest that a better resolution could even be obtained (curve closer to the reference), but unfortunately it is not possible to experimentally verify this point because the Protein I-250 silica-based gel becomes unstable below pH 4.0, according to the manufacturer's directions.

Effect of ionic strength

The influence of c_s on NaPSS calibration curves is a widely studied variable, probably the most commonly used in order to suppress ion-exclusion effects (31-35). Recent contributions have analyzed this variable in both organic and inorganic packings (3-6, 8-10). Briefly, at high enough mobile phase ionic strength, electrostatic repulsive polymer-gel interactions can be significantly decreased or even eliminated, and the polyelectrolyte hydrodynamic volume reduced. Fig. 2C shows the influence of c_s on NaPSS calibration curves at pH 4.0 and for two limit c_p values (0.1 and 10.0 g/L). The higher c_s, the more the curves approach the reference one. Moreover, as c_s increases the divergence between calibrations for c_p=0.1 and c_p=10.0 g/L is reduced. For c_s=0.20 M both curves are practically coincident.

It can be concluded from this variable analysis using a Protein I-250 column for the elution of NaPSS, that at pH 4.0 (lower working limit recommended by the manufacturer) a polyion concentration of at least 10.0 g/L and an ionic strength higher than 0.20 M would be appropriate to minimize the secondary effects, particularly ion-exclusion in this case.

Model

In order to account for the secondary effects of NaPSS observed as a function of c_p, c_s and pH, a model has been developed relating the support

effective pore volume (i.e., the pore volume practically accessible to NaPSS (3)) to the polyion molecular weight. The model incorporates the following elements:

i) An effective pore volume defined in this context in a similar way as in Dubin's model (3) and equivalent to the difference between the total pore volume and the repulsion volume (ion-exclusion volume in ref. 6).

ii) Equations previously developed to correlate the elution volume and distribution coefficients for uncharged polymers in organic media (36-38).

iii) Rather than making use of considerations of the electrostatic potential due to the charged surface of the stationary phase (3), a preferential adsorption coefficient, λ, is introduced here in the framework of polymer solution thermodynamics, recently applied to SEC of uncharged polymers (12,13).

The elution volume for dextrans in Fig. 2 can be expressed as:

$$V_e = V_0 + K_{SEC} V_p \qquad [2]$$

where V_0 denotes the intersticial packing volume, V_p the total pore volume and K_{SEC} the distribution coefficient for a pure SEC mechanism (or fraction of the total pore volume available to the macromolecule). For the Protein I-250 column used in this work, V_0= 5.9 mL and V_p= 6.1 mL, as measured with dextran 500 kD and D_2O respectively. A similar equation for the elution volume of polyelectrolytes can be defined as follows:

$$V'_e = V_0 + K'_{SEC} V_p \qquad [3]$$

V'_e referring to the experimentally measured elution volume for NaPSS which, as can be seen in Fig. 2, is in all cases lower than the corresponding for a dextran of

the same hydrodynamic volume. K'_{SEC} denotes a global partition coefficient taking into account both steric and secondary effects. Thus, K'_{SEC} can be expressed as:

$$K'_{SEC} = K_D K_p \qquad [4]$$

where $K_D(=K_{SEC})$ and K_p account for steric and secondary effects, respectively.

An equation similar to eqn.[3] can be defined specifically for polyelectrolytes in non-ideal conditions (3):

$$V'_e = V_0 + K_{ION} V'_p \qquad [5]$$

If a horizontal line is drawn through any of the pairs of curves in Fig.2 connecting the data points for ionic and uncharged polymers of identical hydrodynamic volume (i.e., the same $\log M[\eta]_{p,c_p,c_s}$ value), an equivalence can be assumed for the distribution coefficients K_{ION} and K_{SEC} defined for the two calibration curves considered. Taking this equivalence into account, the following expression can be obtained from eqns.[3] and [5]:

$$V'_p = K_p V_p \qquad [6]$$

This equation reflects the proportionality between V'_p and K_p (remember that V_p is constant). Since K_p depends on the injected sample molecular weight (12,39), V'_p will also do (3). Thus, the calculation of V'_p for each NaPSS sample in a given set of conditions (c_p, c_s and pH) has been performed as follows:

i) For any given NaPSS molecular weight, an experimental V'_e value is obtained which introduced in eqn.[3] yields the corresponding K'_{SEC}.

ii) From the calibration plots in Fig.2, an elution volume, V_e, is derived from the reference curve corresponding to a hypothetical dextran sample having the same hydrodynamic volume (connecting horizontal line) as the considered

NaPSS sample. When this V_e value is introduced into eqn.[2], a K_{SEC} is obtained for the hypothetical dextran.

iii) Assuming the aforementioned equivalence in these conditions between K_{SEC} and K_{ION} and taking into account eqns.[2], [3] and [5], the following expression can be derived:

$$\frac{K'_{SEC}}{K_{ION}} = \frac{V'_p}{V_p} \qquad [7]$$

Thus, V'_p can be easily calculated from:

$$V'_p = V_p \frac{K'_{SEC}}{K_{ION}} \qquad [8]$$

Just as an example, Table 1 summarizes the V'_p values predicted as described above (together with the V'_e, K'_{SEC}, V_e and K_{ION} values involved in the calculations) for different molecular weight NaPSS samples, at c_p= 10.0 g/L in an acetate buffer (pH 4.0, c_s=0.20 M) as eluent. It can be seen that V'_p decreases as polyion molecular weight increases, in agreement with Dubin's model (3,4). This dependence will be further analyzed in the framework of our model.

If the above reasoning is correct, a congruent calibration curve should be obtained for polyelectrolyte and uncharged samples. Fig.4 depicts the calibrations obtained by plotting log $M[\eta]_{p,c_p,c_s}$ for NaPSS and log $M[\eta]$ for dextran vs. K_{ION} from eqn.[5] at pH values of 5.9 and 4.0, c_s= 0.02 M and for a range of c_p values from 0.1 to 10.0 g/L. A similar "universal" calibration curve has been obtained by Dubin et al. (see Fig.4 in ref.4) for NaPSS (ionic) and pullulan (non-ionic) eluted on controlled pore glass.

TABLE 1

Molecular Weight Dependence of V'_p for NaPSS and Related Magnitudes Involved in its Calculation.

M (kD)	V'_e (mL)	K'_{SEC}	V_e (mL)	K_{ION}	V'_p (mL)
1.6	10.92	0.823	11.0	0.836	6.00
4.0	9.85	0.647	10.3	0.721	5.47
16.0	9.08	0.521	9.95	0.664	4.79
31.0	8.10	0.360	9.15	0.533	4.13
88.0	6.84	0.154	7.35	0.238	3.95

Chromatographic conditions: acetate buffer (pH 4.0; c_S=0.20 M) as eluent; c_p=10.0 g/L.

Let us now introduce some thermodynamic considerations in the derivation of our model. Thermodynamic treatments for non-exclusion interactions have been previously reported for uncharged polymers in organic media (40). Some of these treatments are based on the introduction of Flory-Huggins interaction parameters, χ_{ij} (41), or as more recently proposed, polymer concentration-dependent Flory-Huggins parameters, g_{ij} (12,13). These interaction parameters are related to the distribution coefficient for solute-gel interaction, K_p (36), and the preferential solvation coefficient, λ, which represents the affinity of the solvent (eluent) for either the polymer or the chromatographic support (see

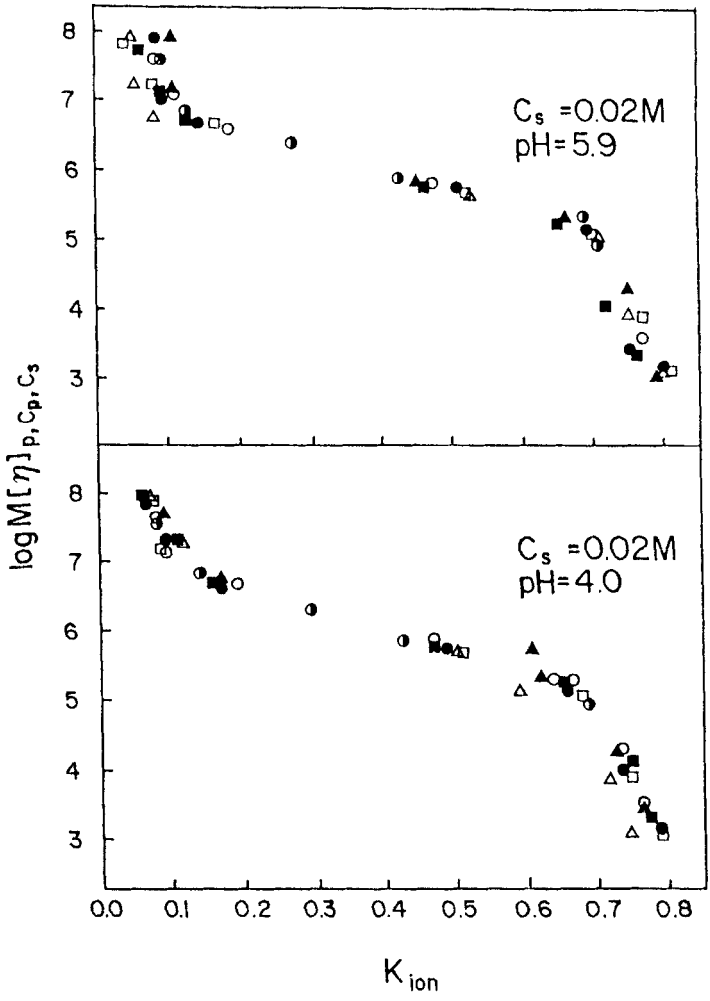

FIGURE 4. Calibration plots, log $M[\eta]_{p,c_p,c_s}$ vs. K_{ION} or K_{SEC}, for NaPSS and dextrans (◑) at different c_p values: (▲) 0.1; (●) 0.5; (■) 1.0; (□) 3.0; (△) 8.0 and (○) 10.0 g/L.

eqns.[25] and [26] from ref.12). Unfortunately, the interaction parameters χ_{ij} or g_{ij} are not known for polyelectrolytes, and therefore, a quantitative evaluation of secondary effects in a similar way as for uncharged polymers cannot be done through K_p and λ. However, it is possible to qualitatively interpret the dependence of V'_p on the polyion molecular weight, as follows. The variation of λ with the molecular weight of the solvated polymer is experimentally given by (42):

$$\lambda M^{1/2} = \lambda_\infty M^{1/2} + A \qquad [9]$$

where λ_∞ denotes the preferential solvation coefficient at infinite molecular mass and A is a constant. On the other hand, λ is proportional to ln K_p (12), K_p being related to V'_p through eqn.[6]. Therefore, the following final relationship can be established:

$$M^{1/2} \ln \frac{V'_p}{V_p} = \frac{\lambda_\infty}{B} M^{1/2} + \frac{A}{B} \qquad [10]$$

B being a proportionality constant.

If the model proposed by eqn.[10] is valid, a plot of the first member of the equation vs. $M^{1/2}$ should yield a straight line. In fact, Fig. 5 shows the plots at two eluent pH values (pH 5.9, upper; pH 4.0, lower part), for c_s=0.02 M and six c_p values in the range from 0.1 to 10.0 g/L. V'_p values were evaluated as mentioned above and illustrated in Table 1. The good correlation observed in all cases undoubtedly validates eqn.[10]. The negative sign of the ordinate values for the plotted data indicates that V'_p is always lower than V_p, which is a general behavior in the present work. This means that the polyelectrolyte is always preferentially solvated by the solvent (eluent) and shows some incompatibility

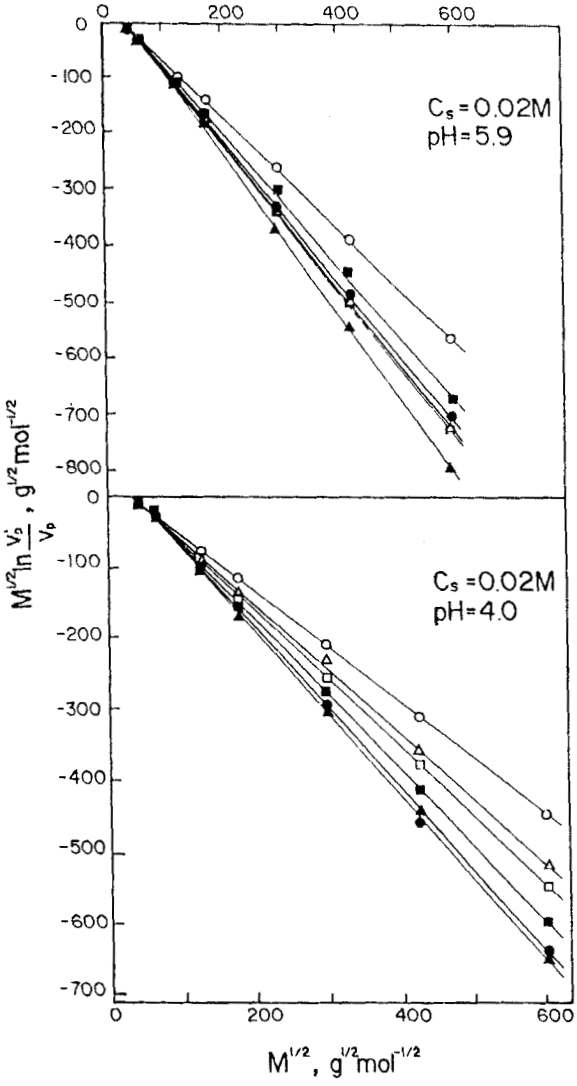

FIGURE 5. Molecular weight dependence of partition coefficient, V'_p/V_p, for NaPSS at different c_p values: (▲) 0.1; (●) 0.5; (■) 1.0; (□) 3.0; (△) 8.0 and (○) 10.0 g/L.

with the support, which results in a certain decrease in the elution volumes with respect to the reference ones.

It can also be observed in Fig.5 that the absolute value of the slope decreases when increasing c_p , which again can be interpreted in the context of the model proposed as a diminution of the preferential solvation of NaPSS by the eluent and a concomitant increase in the compatibility between polymer and support.

On the other hand, note that in the case of dextrans, $V'_p=V_p$, and then the slope of the plot equals zero. This means a complete suppression of ion-exclusion effects and can be interpreted in terms of an identical solvation of the polymer by eluent and support. In fact, it can be seen in Fig. 5 that as c_p increases, the value of the slope approaches zero.

It is worthwhile considering, finally, the meaning (if any) of straight lines exhibiting positive slope values in the $M^{1/2}\ln(V'_p/V_p)$ vs. $M^{1/2}$ plot. Such a result seems *a priori* plausible and should be interpreted in the framework of the present model as due to a preferential solvation (compatibility) of the polyion by the support. Although this situation has not been achieved in the present work using a Protein I-250 column, it is interesting to note that plots with positive slopes have already been obtained from results in our lab for NaPSS in some experimental conditions using other chromatographic supports. This phenomenon can be interpreted in terms of other secondary mechanisms (different from ion-exclusion) such as hydrophobic interactions, etc., and will be presented in more detail elsewhere.

As a conclusion, we have independently analyzed the effect of polyelectrolyte concentration, eluent pH and ionic strength on the

chromatographic behavior of NaPSS and have shown that using a silica-based Protein I-250 column a complete suppression of ion-exclusion effects has not been possible in the range of experimental conditions assayed. Moreover, a novel semi-empirical model relating the support effective pore volume to the polyion molecular weight has been derived from thermodynamic considerations, which qualitatively describes secondary effects (ion-exclusion in the present case) for NaPSS.

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