SIZE-EXCLUSION CHROMATOGRAPHY OF POLYELECTROLYTES

EXPERIMENTAL EVIDENCE FOR A GENERAL MECHANISM

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SUMMARY

The flow of polyelectrolytes through microporous matrices has been studied with several rigid resins including ion-exchange columns in a variety of aqueous buffers differing in composition, pH and ionic strength. The elution of native proteins having well defined structures was measured at very low polymer concentrations in the presence of sufficient supporting electrolyte to exclude kinetic interaction phenomena and Donnan-type interactions amongst the solute molecules. It was found to depend strongly on ionic strength, I, without parallel effects in the bulk properties; the effective radii increased in proportion to $I^{-1/2}$. It is concluded that size-exclusion chromatography (SEC) measures the unperturbed dimensions of a polyelectrolyte which include the surrounding diffuse double layer and any other interactions of the solvent. Chromatography thereby provides a novel approach for the measurement of electrostatic and colloidal properties of macromolecules and of solvation. The bulk viscosity-determined molecular radii, *i.e.*, the size and shape excluding the double layer, are obtained even at low ionic strength either by calibration with similarly charged polyelectrolytes or by extrapolation to high ionic strength. Universal SEC calibration was also achieved on ion-exchange columns. Understanding the physical basis of SEC in view of the results of the present study may provide a new method for characterization of the structure and formation of gel networks.

INTRODUCTION

This investigation aims to present a comprehensive account of the factors that govern permeation of polyelectrolyte macromolecules through microporous matrices. The introduction presents a review of the issues involved, first those that pertain both to non-electrolytes and polyelectrolytes, and then the specific factors that complicate polyelectrolyte behaviour. In the results section, experiments designed to investigate the ion strength dependence of the elution volume of polyelectrolytes of various sizes

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and charges are described. The discussion section starts with a phenomenological summary and then presents an interpretation in the light of polyelectrolyte theory, taking due account of related phenomena like ion exchange and inverse chromatography.

It is generally accepted that the principal molecular parameter governing size-exclusion chromatography (SEC)* is the hydrodynamic volume, rather than the molecular weight. There has been considerable confusion as to the specific description of this volume, in particular its hydrodynamic interpretation using the corresponding Stokes radius (R_s)[3]. While in the biological sciences diffusional friction is considered[4-8], polymer chemists refer to a convective process when employing Benoits universal calibration using the product of the intrinsic viscosity and the molecular weight[9]. To distinguish the latter from the diffusional Stokes radii, I have called it the viscosity radius[10]. A different tradition focuses on the radius of gyration[11] derived from the intrinsic viscosity via the Ptitsyn–Eizner relationship[12]. The radius of gyration is related to a number of other geometric criteria such as the mean end-to-end distance of coiled polymers. Alternatively, entropic arguments have resulted in the proposal that the mean linear extension (L-measure) is the determinant of chromatographic behaviour[13,14]. Other thermodynamic approaches led to the proposal that either the second virial coefficient[15] or even the molecular weight[16] is the factor controlling SEC. The latter proposition thus implies independence from the partial specific volume of the solute. Various notions of perturbed dimensions have persisted, but the extreme proposition of contour length[17] is no longer pursued[4,18] (further references and a discussion of SEC calibration are given in ref. 10). The viscosity radius calibration was modified to account for non-negligible solute concentrations in semi-dilute solutions[19]. On the whole, the reported deviations from an universal calibration seem to be due to secondary non-SEC effects such as adsorption to the matrix and solvent partitioning[20-22]. Mixed solvents are particularly prone to preferential solvation of the matrix by one of the components[23]. Secondary effects may be reduced by optimizing the matrix surface for the specific solute and solvent properties[24]. Identical calibrations have been claimed for aqueous and organic solvents[25], however, it is known that even different organic solvents yield different calibrations of the same column[23,26-28]. In those calibrations the void volumes remained the same and thus different pore sizes were invoked to explain the results[26]. The pore-size changes were thought to be due to different degrees of solvation of the matrix as a function of the solvent strength[27]. Thus porosity measurements differ for different solvents and should depend on the method used (N_2-BET, mercury, solvent removal by centrifugation)[28-30]. Some differences however originated from an erroneous model of all-or-none pores[8,31,32]. The error-function calibration of SEC is based on this very model. Instead a gel seems to contain a dominant pore size with a diminishing series of larger ones related perhaps by fractal properties of porous solids.

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* SEC has been recommended by polymer chemists as the appropriate terminology[1,2] rather than gel permeation chromatography (GPC), gel filtration, molecular sieve chromatography, etc. In the biological sciences, however, GPC is still used.

Composite matrices then are sums over such integral distributions and the elution volume is of the general form

$$V(\text{ml}) = \sum_i \int_j v_{ij} \left(1 - \frac{R}{r_{ij}}\right)^x$$

where R is the effective radius of the solute, r_{ij} the pore radius of the ij type of pores, v_{ij} the total volume of the ij type of pores, and $x \approx 1.5$–2.0. In practice the integral has been replaced by a summation.

Systematic investigations of aqueous SEC and polyelectrolytes[25,26,33−68] have been devoted to various synthetic polymers both in aqueous solutions[26,33,37,43] and dimethylformamide[25,34,38,42,44,52], aqueous solutions of dextran[25,26,33,37,43,61], lignin[45], latex[35,55], small ions[40,56,57,67,68], nucleic acids[51], native proteins[39,41,43,46−50,53,54,61,62,65] and proteins denatured by sodium dodecyl sulphate (SDS)[48,64]. The key issue in the study of polyelectrolytes clearly has been the role of counter ions and thus ionic strength* (see refs. 37 and 47). Polyelectrolytes are a classic example of rapidly interacting systems[69] and their pattern of SEC elution in the absence of a supporting electrolyte[25,34,36,45,52,56−58,63,66] may be understood in terms of the general chromatographic principles of interacting systems[7,8]. These kinetic complications are suppressed if (1) the dialysate of the sample is used as the column eluent, and (2) the contribution of the polyelectrolyte to the total ionic strength is negligible[60]. Even under such dilute polymer conditions the intrinsic viscosities of polyelectrolytes that are measured in bulk solution depend on the ionic strength[26,33,42,44,45,63]. These ionic strength effects on the macromolecular size have been interpreted in part as due to the swelling of the polyelectrolyte coil[70], but were also ascribed to double-layer effects[71,72], *i.e.*, the preferential binding of macromolecules not only by water but also by the supporting electrolyte. Native proteins, which have a compact folding state and which may be monitored by a variety of techniques to probe conformational changes and denaturation, offer clear advantages over flexible polymer coils in the study of SEC charge effects. While one report on polyelectrolyte coils observed universal calibration at all ionic strengths as long as the correct bulk intrinsic viscosity was employed[45], others reported the ionic strength dependence of SEC to be larger than could be accounted for by the ionic strength-dependent changes in shape observed in bulk solution[33,38,42,52]. These differences between the bulk and chromatographic charge effects have been ascribed to secondary ion-exchange mechanisms due to residual charges on the matrix[37,39,54,56,59,64] but also to the binding of salt or solvent to the polyelectrolyte[44,52]. Delayed elution or complete adsorption of cationic polyelectrolytes to SEC matrices is well documented[10,36,39,50,53,59,62], but the possibility of hydrophobic effects[61] is difficult to

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* Ionic strength is operationally defined in molar units as:

$$I = \frac{1}{2}\sum c_i z_i^2$$

where c_i is the concentration and z_i the charge of the i-th ionic species. Ion-specific variations clearly operate even at moderate ionic strength (see also ref. 61) and more specific interactions between polyelectrolytes and multivalent counter ions may occur at very low concentrations.

eliminate. In one case, delayed protein elution was ascribed to Ca^{2+}-induced conformational changes leading to hydrophobic effects[65]. Direct evidence for residual negative charges on the matrix was obtained by titration[51] and electrophoretic mobility[61], and the number and pH dependence of such charges varies widely amongst different matrix brands[10,43]. Adsorption of ions[67] may further increase the effective surface charge density. All of the above effects can be suppressed in the presence of sufficient supporting electrolyte, and an universal calibration can then be obtained for charged and neutral macromolecules[10,36], even though conflicting claims do exist[33]. Published data demonstrate that elution of proteins at lower ionic strength also depends on the pH and is related to both the pI values of the native protein and the protonation constants of the matrix[50,53,61,62]. Regardless of ionic strength, SEC elution has been correlated with macromolecular size at least at alkaline pH[47,60,73], albeit the column calibration differs from those obtained under normal or high salt conditions.

I have previously studied well defined monodispersed biopolymers both in free solution and in several different matrices in the presence of normal-to-high levels of a supporting electrolyte[10]. These data exclude the contour length, L-measure, radius of gyration, diffusion coefficient, molecular weight or second virial coefficient as a single determinant of elution. However, excellent correlation is obtained with the shape function from the intrinsic viscosity, independent of the matrix and thus presumably of the pore geometry. No flow-rate dependency was observed above 10 μl/min. In the present report these observations are extended to solutions of low ionic strength. Variation of the ionic strength is used to demonstrate that macromolecules that permeate a microporous network of comparable size actually have properties that differ from those in bulk solution. As part of an effort to develop a comprehensive theory of SEC, the key role of the diffuse double layer around polyelectrolytes in the elution pattern in SEC will be addressed. It will be demonstrated that this layer depends on the charge and geometry and for an highly charged species the size of the double layer is found to coincide with the Debye length only for a point-charge geometry*. It will be proposed that this counter-ion cage surrounding the polyelectrolyte is impenetrable even in a neutral, pore-forming matrix**. Ion exclusion will

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* Strictly speaking the Debye–Hückel parameter κ is an integration constant of the Poisson equation with units of reciprocal length. For a planar surface and low surface charge, the Debye length, κ^{-1}, corresponds to a $1/e$ drop in the electrostatic potential, Ψ. The Debye length coincides with the distance of the "plates" of an equivalent condenser as long as the surface charge is low, and this definition also holds for a spherical geometry[74]. The Debye length is only one of the factors that determines the region of solution around the polyelectrolyte whose properties differ from those of the bulk. The present paper defines the thickness of this region in absolute terms of energy. In principle the double layer extends to infinity, in practice one defines the average thickness with respect to kT (k = Boltzmann constant; T = absolute temperature), *i.e.*, where the potential has dropped below the thermal pressure. The equilibrium distance of two polyelectrolytes in a medium of supporting electrolyte thus offers an operational means to define the thickness of a double layer (see Discussion).

** In deriving the role of the diffuse double layer one starts with Coulomb's law and notices that the effective charge decreases with separation due to the distribution of the shielding counter ions, *i.e.*, the double layer. The double layer is impenetrable for polyelectrolytes of equal sign only in a statistical mechanical sense. Oppositely charged particles may replace the counter ions and penetrate the double layer mainly due to the concomitant entropy gain. In order to penetrate, neutral molecules of finite dimensions need to replace counter ions, requiring electrostatic work to be performed which makes the double layer impenetrable in the above sense even for neutral macromolecules. The diffuse double layer should not be viewed as a rigid body or a mechanical barrier around a polyelectrolyte.

be considered as part of SEC proper and is distinguished from the entirely different mechanisms that govern charge attraction, *i.e.*, ion-exchange chromatography (IEC). I shall discuss the prospects of chromatography to elucidate polyelectrolyte properties and also provide practical guidelines for the measurement of polyelectrolyte size and shape by SEC. It is concluded that the mechanism of separation in SEC depends on the unperturbed dimensions of the molecule which include the bulk viscosity radius, the diffuse double layer and any other solvent interactions with the macromolecule. However, the peak dispersion is governed solely by the bulk viscosity radii.

EXPERIMENTAL

Chromatographic instrumentation

The high-performance liquid chromatography (HPLC) set-up included an inert glass pump (Pharmacia P500), an injection valve (Rheodyne 7125), a variable wavelength UV monitor (Knauer 731.87), a dual-channel recorder (Metrawatt SE120), narrow-bore PTFE tubing (Bemu, Krefeld, F.R.G.) and prepacked aqueous SEC columns.

Three TSK 5000PW (Toyo-Soda/LKB) 60-cm stainless-steel columns[43,75] were operated at a flow-rate of 250 μl/min. According to titration studies this matrix contains the fewest residual negative charges of all commercially available columns[10,51]. Corrosion by the aqueous, salt-containing eluent unfortunately is a major drawback compared to Superose columns which are available in inert glass packings[76]. Most reported data were obtained with the same column, TSK 5000PW, that had been previously exposed to several hundred injections. A brand new column, TSK 5000PW-3, was used to assess column aging. Preliminary results were obtained with a third column, TSK 5000PW-1 (previously used in ref. 73).

A TSK 6000PW (Toyo-Soda/LKB) 60-cm stainless-steel column was operated at a flow-rate of 150 μl/min. A Superose 12 (Pharmacia) 30-cm glass column[77] was operated at a flow-rate of 500 μl/min. A Mono-S HB10/10 (Pharmacia) polystyrene based wide-pore strong cation-exchange resin packed in a 10-cm glass column was operated like a SEC column at a flow-rate of 250 μl/min. Both the PW resin[78] and the agarose gels[79] mostly contain flow-through capillaries as judged from scanning transmission electron micrographs. For a summary of the column properties and references see ref. 10.

The eluent buffers were continously saturated with helium and filtered through 5-μm porous PTFE (LKB 2135). The injection volumes were 5 μl at a protein concentration of 1–10 mg/ml. The general procedures and methods of analysis have been described previously[10]. All measurements were done at the lowest possible concentrations, particularly at lower ionic strengths. Within measurement error, these data correspond to the limit of infinite dilution, which was verified by extrapolating a series of concentrations at injection volumes up to 200 μl. Initial experiments indicated that with small injection volumes it was not critical to match the injected sample to the ionic strength of the column when there were comparatively large amounts of supporting electrolyte present in the eluents. Thus dialysates need not be used as column eluents for the present purposes. All data were obtained from a random sequence of eluent conditions including several repetitions at later times. In this manner, aging effects of the matrix do not bias any of the reported observations.

TABLE I

REFERENCE DATA

Protein	$R_s(nm)$*	pI	$z_{5.0}$**	$z_{8.0}$**	Ref.
MS2	13.9				10
Thyroglobulin	8.6	4.5	≈ -30	≈ -270	10, 80
Ovalbumin	2.8	4.7–4.9	≈ -1	≈ -13	10, 81, 82
Calmodulin	2.1	3.9–4.3		≈ -26***	83
Myoglobin horse	1.9	7.3		≈ -1	10, 81
Myoglobin sperm whale		8.2	$\approx +8$	≈ 0	81, 84
Myoglobin sperm whale (minor variant)		7.7		≈ 0	81

    * Stokes radius calculated from the diffusion coefficient.
    ** Titrated charge relative to the isoelectric point at different pH values as indicated.
    *** Calculated from the primary sequence.

Materials

MS2-Phage was a gift from Christoph Biebricher, calmodulin from bovine brain was kindly purified by Wolfgang Koch. Other proteins were commercially available and used without further purification: myoglobin (horse, Sigma M1882), myoglobin (sperm whale, Sigma M0380), ovalbumin (Sigma A7641), thyroglobulin (Sigma T1126), vitamin B_{12} (Sigma V2876). The 754 bp DNA restriction fragment was a gift from Stephan Diekmann. Universal calibrations of the columns were performed with the samples and methods given in ref. 10. Further reference data are compiled in Table I.

Buffers

Buffers were freshly made from analytical grade chemicals and water distilled from quartz. Ionic strength and pH values were calculated from tabulated pK values, corrected for ionic strength by the Davis equation[85], and verified by conductivity (LF20 by WTW, Weinheim, F.R.G.) and pH measurements (PHM64, Radiometer, Copenhagen, Denmark) at 20°C. The large temperature coefficient of the pK of Tris necessitated special care in the preparation of Tris-containing buffers.

Circular dichroism

Circular dichroism (CD) spectra in the far-UV (200–240 nm) were measured with a Jobin-Yvonne Mark V (Instruments SA, France) to monitor the native secondary structure of the proteins under the diverse solution conditions.

Electrophoresis

Proteins were also studied by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the Tris system and by isoelectric focusing (IEF)[86].

RESULTS

Differential solvation effects

Fig. 1 shows the retention volume of the neutral molecule vitamin B_{12} (co-
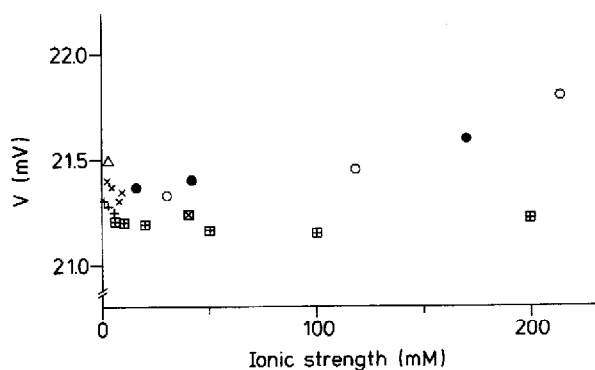
Fig. 1. Retention volume of vitamin B_{12} in different aqueous buffers on a TSK 5000PW column. Eluents: sodium phosphate at pH 7.2 (○) and 6.6 (●); 2-morpholino ethane sulphonic acid (MES) at pH 6.4 (△); Tris–HCl at pH 8.0 (+) and 7.2 (×); sodium chloride + 10 mM Tris–HCl at pH 8.0 (⊞) and 7.2 (⊠). All buffers contained 0.4 mM 2-mercaptoethanol (BME).

balamin) in different eluents. Its size is small compared to the separation range investigated and thus measures the total inclusion volume, V_{TOT}, of a column. It is well known that the classic chromatographic media, like soft Sephadex gels, swell due to osmotic pressure effects and this has prevented systematic comparisons of elution
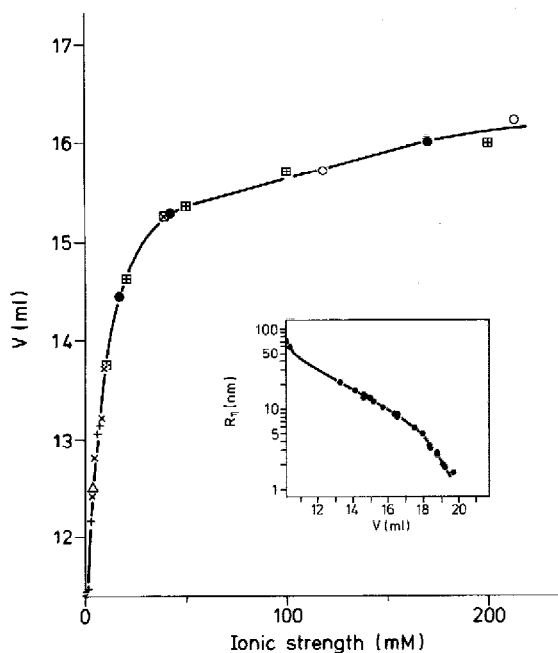

Fig. 2. Retention volume of thyroglobulin as a function of the ionic strength in different aqueous buffers. The TSK 5000PW column at a flow-rate of 250 μl/min was used. Eluents as in Fig. 1. Insert: universal calibration at high ionic strength (100 mM sodium phosphate pH 7.0) using the reference standards listed in Table II of ref. 10.

patterns under different conditions. Modern HPLC columns on the other hand contain rigid porous matrices and any change in the effective pore dimensions seem to be related to different degrees of solvation, both of the matrix and the macromolecule (see Introduction). Larger macromolecules indeed show less buffer-to-buffer variation at any given pH and ionic strength (Fig. 2). Different degrees of solvation of the solute rather than of the matrix may well dominate the observed effects with small molecules like vitamin B_{12}. The possibility of phosphate-induced adsorption cannot be excluded however. The differences between a variety of typical aqueous biological buffers at the same ionic strength seem to be negligible for the large number of proteins tested in the entire size range of interest (data not shown except for Fig. 5). The extent to which calibrations in aqueous and different organic solvents may be compared remains to be established.

Concentration effects

From a series of concentrations (data not shown) the limit of infinite dilution was established. Retention increased at increasing concentrations, *i.e.*, the effective radius of the macromolecule decreased. This effect is due to the second virial coefficients of like solute molecules[87,88] which may be large at low ionic strength due to Donnan terms[60]. The effect is equally present in bulk solution. All data reported in the present investigation were obtained at sufficiently low concentrations to correspond approximately to infinite dilution within the errors of measurement. Under these circumstances measurements of the intrinsic viscosity and diffusion coefficient become independent of the ionic strength in bulk solution as long as conformational changes do not take place. The preservation of the native state was verified by far-UV circular dichroism. The SEC peaks remained symmetrical and further peaks did not arise, indicating lack of aggregation. The large ionic strength effect observed for the 670-kilodalton thyroglobulin dimer in the microporous matrix (Fig. 2) therefore is not paralleled by bulk properties. It is not due to a conformational change as suggested previously[46].

Influence of pH and ionic strength

Different myoglobins were selected to illustrate the effect of the net charge on elution (Fig. 3). Sperm whale myoglobin was chosen since it retains the fully native conformation even at pH 5.0[84]. Horse myoglobin exists in native conformation both below and above the pI so that cationic and anionic but otherwise identical species may be studied. Measurement of different pI variants at the same pH eliminates variation of residual matrix charges. Fig. 3 demonstrates that the elution of all cationic species was progressively delayed as the ionic strength was decreased. This effect increases with the net positive charge (*cf.*, ref. 53) and eventually results in complete adsorption, *i.e.*, a fully developed ion-exchange mode. All negatively charged species were progressively eluted sooner as the ionic strength was decreased (*cf.*, Fig. 2). Polyanions with nearly zero net charge, such as the pI 7.7 variant of sperm whale myoglobin at pH 8.0, were eluted sooner at intermediate ionic strength but eventually became retarded at very low ionic strength. This may indicate either that the elution of polyelectrolytes close to their isoelectric points is not determined by the net charge but instead by some higher moment of surface charges, or that the pI changes with ionic strength or that the protein was denatured upon adsorption. Adsorption is
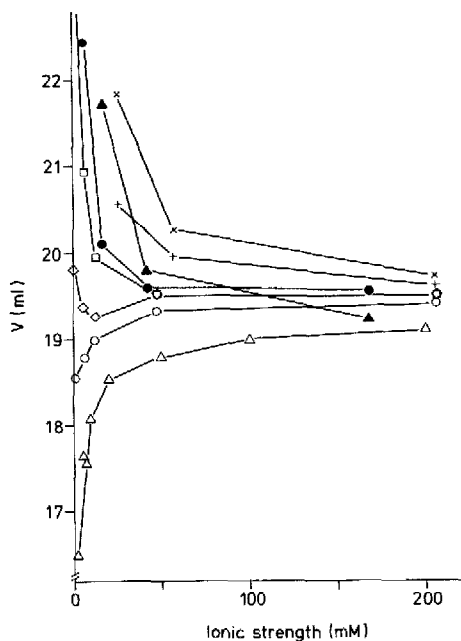
Fig. 3. Retention volume of myoglobin as a function of the ionic strength using two different columns, TSK 5000PW-3 (O, ●, +, ×, □, ◇) and TSK 5000PW (△, ▲). The former was not used before obtaining the data for this figure; the latter had been previously exposed to some 300 injections and was shelved in aqueous sodium azide. Samples: horse myoglobin (pI = 7.3) in Tris–HCl both with and without sodium chloride at pH 8.0 (O, △), in sodium phosphate buffer pH 6.6 (●, ▲) and in sodium chloride + 10 mM sodium acetate at pH 5.0 (+); sperm whale myoglobin (pI = 8.2) in sodium chloride + 10 mM sodium acetate at pH 5.0 (×) and in sodium chloride + 10 mM Tris–HCl at pH 8.0 (□); sperm whale myoglobin (pI = 7.7) in sodium chloride + 10 mM Tris–HCl at pH 8.0 (◇). Vitamin B$_{12}$, which is to correspond to V_{TOT}, is eluted 0.2 ml later on TSK 5000PW-3 compared to Fig. 1 showing TSK 5000PW.

paralleled by a decrease in sample recovery at low ionic strength for proteins near their isoelectric points (data not shown). The recovery of charged proteins on the other hand increased with decreasing ionic strength (*cf.*, ref. 46). Fig. 3 also shows that even a brand-new column bears some residual charge that binds polycations. Both effects, repulsion from and adsorption to the matrix, increase as the column ages.

In addition to thyroglobulin (Fig. 2) and the myoglobins (Fig. 3), a number of other proteins were investigated as a function of ionic strength. All elution volumes were converted into effective viscosity radii, R, via an universal calibration graph obtained at high ionic strength, I (Fig. 2, insert). An excellent linear correlation between R and $I^{-1/2}$ was obtained (Fig. 4) but not with I^{-1} as would be expected for the Donnan contribution to the second virial coefficient. Since the corresponding viscosity radii in bulk solution are independent of the ionic strength (see above), the observed effect must originate from an interaction of the polyelectrolyte sample with the microporous matrix. This interaction reflects the increasing size of the diffuse double layer both around the macromolecule and the pore surface as the ionic
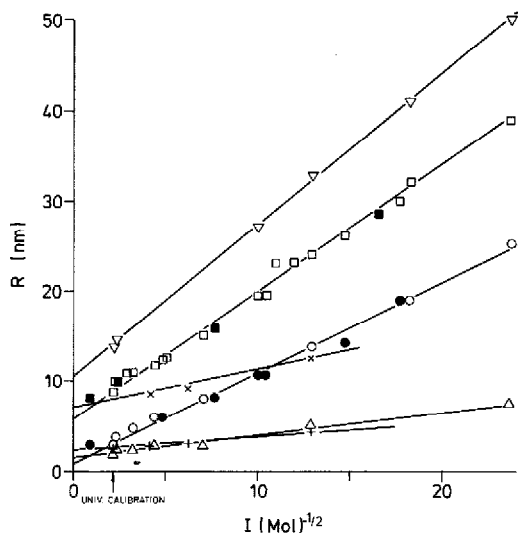
Fig. 4. Size of the counter-ion layer surrounding the polyelectrolyte according to SEC analysis on a TSK 5000PW column. The viscosity radii were obtained with the universal calibration at high ionic strength (Fig. 2, insert) and the observed variation correlated with the ionic strength. Measurements were done in a variety of buffers all containing 0.4 mM BME: MS2 at pH 8.0 ($\triangledown$); thyroglobulin (pI = 4.5) at pH 8.0 ($\square$), 6.6 ($\blacksquare$) and 5.0 ($\times$); ovalbumin (pI = 4.7–4.9) at pH 8.0 ($\bigcirc$), 7.2 ($\bullet$) and 5.0 (+); horse myoglobin (pI = 7.3) at pH 8.0 ($\triangle$). R is the total radius of the macromolecule plus the double layer.

strength is reduced*. The elution tended to become independent of the ionic strength above 300–500 mM.

Thyroglobulin at pH 6.6 and 8.0 yields identical ionic strength dependencies in spite of different net charges (Fig. 4). So does ovalbumin at pH 7.2 and 8.0. Both, however, behave quite differently at pH 5.0 which is close to their isoelectric points (see Table I). Calmodulin, furthermore, is eluted in a similar manner to ovalbumin at pH 8.0 (data not shown, cf., Fig. 7) even though it bears twice the net charge. Highly charged molecules are thus eluted nearly independently of the net charge. This demonstrates that the diffuse double layer which surrounds polyelectrolytes approaches a maximum size. Further counter ions then add mostly to the innermost region, described by the non-linear Poisson–Bolzmann equation, and to the Stern layer (see Discussion)[71]. This relates to an effect referred to as the counter ion condensation[89]. Once the net charge decreases below some condensation threshold, the diffuse double layer itself diminishes and the slopes in Fig. 4 decrease for a given molecule. A detailed correlation between the net charge and the dimensions of the diffuse double layer requires simultaneous titration studies of high precision since the isoelectric points themselves depend on the conditions[81,82]. In the case of weakly

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* The square-root dependency on ionic strength stems from the integration of a one-dimensional, Poisson–Boltzmann distributed, diffuse double layer with a linearized exponential term. It is independent of surface geometries such as spherical (Debye, Hückel), planar (Gouy, Chapman, Stern) or cylindrical. In large part, the size of the double layer will be determined by the outer region of low charge density warranting linearization and neglect of the complex geometries close to a polyelectrolyte surface.

charged small molecules a good correlation of the elution volume and net charge has been observed[40]. It is however obvious from Fig. 4 and Table I that the net charge required for saturation increases with the size of the macromolecule, as anticipated if the surface charge density and possibly the curvature of the macromolecule surface determines saturation. Fig. 4 also demonstrates that the maximum dimension of the diffuse double layer at a radius of 8.6 nm (thyroglobulin) is 1.5 times larger than at a radius of 2.8 nm (ovalbumin). The maximum double layer size continues to increase for larger macromolecules (MS2 and unpublished data). Peak widths, a measure of dispersion, only increase with increasing bulk viscosity radius, R_η, and remain roughly independent of ionic strength (data not shown). This indicates that the ionic strength changes the volume accessible to the macromolecule in the matrix but does not effect its mobility.

Calibration at low ionic strength

In the previous section all elution volumes were interpreted by means of an universal calibration at high ionic strength regardless of the actual ionic strength of measurement. The radii thus obtained are interpreted as being comprised of the actual radius of the macromolecule as measured in the bulk plus the diffuse double layer. Alternatively, elution volumes were also correlated directly with R_η at each ionic strength. Data for the TSK 5000PW column are not replotted in this manner. Instead, Fig. 5 presents the equivalent data obtained on a TSK 6000PW column. It is seen that, at pH 8.0, different universal calibration graphs were obtained even at low ionic strength with MS2, thyroglobulin, ovalbumin and numerous other samples, but not with myoglobin or immunoglobulin G (IgG) for example. It is obvious that samples of the same R_η but different slope, $dR/dI^{-1/2}$, may not fall on the same
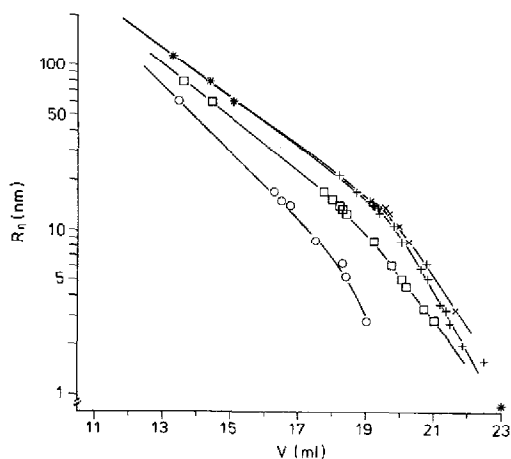

Fig. 5. Direct calibration of a TSK 6000PW column at different ionic strengths with the R_η values of some of the compounds listed in Table II of ref. 10. Eluents: 8 mM sodium phosphate, 1 mM sodium salt of EDTA pH 6.85, 179 mM sodium chloride (total $I = 200$ mM) ($\times$); 40 mM sodium borate pH 8.0, 0.4 mM BME, 58 mM sodium fluoride (total $I = 60$ mM) ($+$); identical calibrations were obtained with 8 mM sodium phosphate, 1 mM sodium salt of EDTA, pH 6.85, 37 mM sodium chloride (total $I = 60$ mM); 20 mM Tris–HCl pH 8.0 (total $I = 12$ mM) ($\square$); 40 mM sodium borate pH 8.0, 0.4 mM BME, 2 mM sodium fluoride (total $I = 4$ mM) ($\bigcirc$).
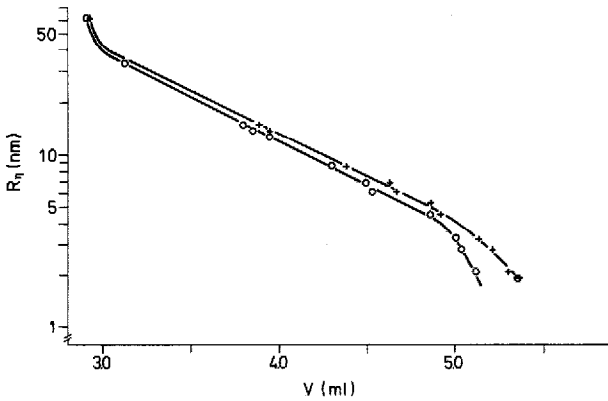
Fig. 6. Universal size exclusion calibration of the strong cation-exchange column Mono-S using some of the reference standards listed in Table II of ref. 10 and in addition a 754 base pairs (bp) DNA restriction fragment (R_η = 34 nm). Flow-rate: 250 μl/min. Eluent: 10 mM Tris–HCl pH 8.0 adjusted with sodium chloride to a total ionic strength of 200 (O) and 600 mM (+).

calibration graphs at low ionic strength. Fig. 5 is constructed with samples of equally high net charge.

Role of the matrix charge

To investigate the role of the residual charges on the matrix a strong cation-exchange column (Mono-S) was studied for comparison. Fig. 6 illustrates that even an ion-exchange column provides a satisfactory universal SEC calibration at high ionic strength, albeit higher salt concentrations had to be used to approach the limit calibration than are required for typical SEC columns with only few residual charges.
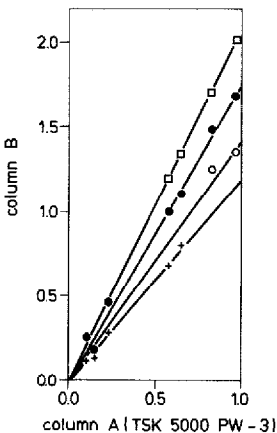

Fig. 7. Correlation diagram of the ionic strength variation $dR(nm)/dI^{-1/2}(M)$ among different columns. Data are taken from the slopes of plots similar to the one shown for TSK 5000PW (Fig. 4). Columns: A = TSK 5000PW-3; B = Mono-S (□), TSK 5000PW (●), TSK 6000PW (O) and Superose 12 (+). From left to right the data are for horse myoglobin at pH 8.0 ($z \approx -1$), ovalbumin pH 5.0 ($z \approx -1$), thyroglobulin pH 5.0 ($z \approx -30$), ovalbumin pH 8.0 ($z \approx -13$), calmoldulin pH 8.0 ($z \approx -26$), thyroglobulin pH 8.0 ($z \approx -170$), MS2 pH 8.0. The Debye length corresponds to a slope of $dR/dI^{-1/2} = 0.3$.

This column also displayed an elution volume dependence on ionic strength that gave a linear relationship between R and $I^{-1/2}$ like that shown in Fig. 4 (data not shown). A comparison of the slopes, $dR/dI^{-1/2}$, is shown in Fig. 7. The same procedure was followed for three other columns. Fig. 7 illustrates that the brand-new TSK 5000PW-3 column exhibited the smallest charge effect. Next best was Superose 12, followed by TSK 6000PW, TSK 5000PW and Mono-S. Both TSK 6000PW and TSK 5000PW had been in frequent service prior to this comparison. Fig. 7 shows that the different columns scale proportionally and differ at most by a factor of 2 even though the number of charges on the matrix differs by orders of magnitude. This saturation of the matrix double layer is equivalent to the observations regarding polyelectrolyte charge reported above.

DISCUSSION

Calibration and analysis procedure for SEC of polyelectrolytes

The only correct data on the macromolecular size of a polyelectrolyte proper, *i.e.*, independent of its surrounding counter-ion double layer, are obtained at very high ionic strength, since the thickness of the double layer then becomes negligibly small. If lower ionic strengths must be employed for reasons of stability, conformation or aggregation state, the measurements should be performed at a series of ionic strengths, converted into overall radii via an universal calibration graph, ideally obtained for infinite ionic strength and the data extrapolated to $I^{-1/2} = 0$. In practice, satisfactory results are obtained by universal calibration at $I = 100$ or 200 mM (see ref. 10) and ionic strength extrapolation to these values rather than to infinity (see Figs. 2 and 4). The slope of this extrapolation on the other hand provides an important insight into the polyelectrolyte nature of the substance under investigation.

However, useful estimates of R_η can be obtained by calibrating the column at a single ionic strength also employed for the sample measurement, but only for sample and calibration substances with saturated diffuse double layers, *i.e.*, at or above the condensation threshold. Fortuitously, all of the proteins that were originally employed for this direct calibration at low ionic strength (at pH 8.0[60,73] and pH 8.5[47]) were sufficiently negatively charged to have saturated double layers. The systematic errors introduced in this manner decrease with increasing ionic strength but are still present as second order effects in the buffers of $I = 100$–200 mM conventionally used for SEC analysis. This applies in particular to sets of data that were not preselected for uniformly high negative net charge (see Fig. 4). It may explain in part the systematic differences observed between the radii of globular proteins determined by SEC and those obtained in bulk solution[90,91].

Since the counter-ion double layer is additive to the size of the macromolecule (Fig. 4) rather than multiplicative[42], the elution volume is not linearly related to $I^{-1/2}$ (*cf.*, refs. 25 and 39). The systematic errors that are introduced in the universal calibration at finite ionic strength necessarily diminish with increasing size of the macromolecule.

Determination of molecular weights

In most cases, reliable molecular weights are obtained by combining the sedimentation coefficient, s, and the (extrapolated) elution radius, R_η[10]. A good approximation for proteins is

$$M_w \text{ (kilodalton)} = 4.3 \, s(\text{Svedberg}) \, R_\eta(\text{nm}) \tag{1}$$

provided that R_s (Stokes radius) $\approx R_\eta$, which is the case if $R_\eta^2/s < 3$ (refs. 10 and 60). If the shape and hydration are known, molecular weight estimates may be derived solely from the R_η values. For coiled polymers, like most synthetic polymers and proteins denatured in 6 M guanidine hydrochloride, R_η values may be converted into molecular weights via the Mark–Houwink relationship[92]:

$$R_\eta^3 \approx [\eta] \, M = cM^{1.66+\varepsilon} \tag{2}$$

where $[\eta]$ is the intrinsic viscosity and the factor ε measures coil expansion, *i.e.* deviation from ideal Gaussian coil polymer conformation. It then suffices to determine independently the Mark–Houwink coefficients, c and ε, which differ for each class of polymer. Aqueous SEC may be calibrated by readily available monodisperse biopolymers. This is actually simpler than classic calibration with certified synthetic polymer standards which is still the only method available for non-electrolytes in organic solvents. By taking into account the ionic strength dependence of universal calibration for polyelectrolytes, it is even possible to calibrate in different eluents, *e.g.*, using native proteins or viruses in the absence of denaturant in order to use the same column to determine single polypeptide chain molecular weights in 6 M guanidine hydrochloride[93].

Preparative strategies

Regnier[94] has emphasized the preparative potential of low ionic strength to improve resolution and separate isoelectric variants. With the better insight now available, separation strategies may be derived from first principles: on negatively charged matrices the optimum pH is as close to the pI of the more basic of two overlapping components as is compatible with yield. Ion-exchange columns may provide even superior SEC resolution (as originally proposed by Crone[40,68]) and are also available as anion-exchange resins suitable for polycations. Alternatively, one may choose a pH between the pI values of the two overlapping components and exploit adsorption of the more basic component (see Fig. 3). The counter-ion cage protects macromolecules efficiently from adsorption to the matrix, thus stabilizing labile aggregates that might dissociate in conventional IEC, reversed-phase chromatography or IEF. It also prevents conformational changes and loss of activity. Loss of yield at increasing ionic strength is related to the salting out effect in aqueous reversed-phase chromatography (hydrophobic interaction chromatography).

Column aging

With increasing use, the adsorption of cationic polymers was found to increase and polyanions were eluted sooner (Fig. 3) thus shifting the universal calibration graphs even at 100–200 mM ionic strength. Whether this is due to trapped polyelectrolytes from previous injections or derivatization of the column by sodium azide during storage has not yet been investigated. Calibrations were therefore repeated periodically. These observations suggest that the service lifetime of a column, the precision and comparability are increased by routinely reporting viscosity radii in addition to retention volumes.

Mechanism of SEC

Molecular properties are usually defined for infinitely dilute particles and concentration effects due to their mutual interaction have traditionally been expressed as second and higher order virial coefficients. The matrix wall–solute interactions vanish once the pore size becomes significantly larger than the solute macromolecule, *i.e.*, the matrix wall approaches infinite dilution and conditions of bulk solution become established. Additional solute–solute interactions, present both in chromatography and in bulk solution, vanish at sufficiently low solute concentrations. The present investigation demonstrates that solute–matrix interactions of polyelectrolytes crucially depend on electrostatic forces. At thermal equilibrium, charge attraction is a short-range force that leads to binding, *i.e.*, conventional IEC behaviour[95], where the matrix presumably penetrates the counter-ion cage that surrounds the polyelectrolyte. In the case of charge repulsion, this penetration is energetically unfavourable and the surfaces are separated over large distances by the diffuse double layers, whose size is proportional to the Debye length, κ^{-1}, which is proportional to $I^{-1/2}$. For example, the binding of cations and exclusion of anions from a given matrix are not reciprocal mechanisms. The Donnan contribution to the second virial coefficients alone depends on I^{-1}.

An intuitive understanding of the Coulomb interaction between polyelectrolytes in the presence of a shielding supporting electrolyte is provided by considering the potential surrounding each polyelectrolyte. The potential around a spherical particle is given in the limit of linearization by[71]

$$\Psi = \Psi_s \cdot \frac{a}{x + a} \cdot e^{-\kappa x} \tag{3}$$

where Ψ_s is the linear double layer potential, a is the radius of the particle and x is the distance from the surface. For low charge densities Ψ_s equals the true surface potential. As the charge density increases, Ψ_s increases less than the true potential at the surface of the particle. This is an immediate consequence of the linearization of the exponential counter-ion distribution assumption on which eqn. 3 is based. It means that counter ions increasingly are found close to the surface and do not affect the total dimension of the double layer which is dominated by its outer part. Thus Ψ_s seems to saturate but will never strictly do so as exemplified by the slightly increased value of $dR/dI^{-1/2}$ for calmodulin compared to that of ovalbumin. Furthermore, the true surface potential progressively increases less as the surface charge density increases. This saturation of the true surface potential has been verified experimentally[96]. In consequence, particles of equal size will be eluted in an identical manner nearly independent of charge except for low charge densities.

Eqn. 3 also features the qualitative dependence on particle size. As the particle becomes larger its curvature approaches a flat double layer, $a/(x + a) \rightarrow 1$, which increases Ψ and thus the dimension of the double layer. This provides a qualitative explanation of the experimental observation that smaller globular macromolecules exhibit smaller slopes, $dR/dI^{-1/2}$, even at saturated values of Ψ_s. A future study of the influence of di- and multivalent counter ions (*cf.*, ref. 71) may help clarify whether this explanation suffices. The actual determinant of repulsion is the interaction energy between the polyelectrolyte macromolecules and the pore wall. In the case of weak

interactions, the repulsion energy becomes proportional to the product of the potentials Ψ. The interaction energy between two polyelectrolyte spheres is given by[71]

$$V(x) = c\Psi_{s1}\Psi_{s2} \cdot \frac{a_1 a_2}{x + a_1 + a_2} \cdot e^{-\kappa x} \tag{4}$$

where x is the distance of closest approach of the two spheres. Eqn. 4 shows again the correct trend but should not be expected to account for the actual case whose simplest model is that of a sphere within a cylinder. Since Bessel functions can be approximated by exponential functions in certain cases, the solution applicable to SEC is expected to have the same general form but with a different pre-exponential factor. Fig. 7 demonstrates the predicted multiplicative influence of the size of the macromolecule.

The slope, $dR/dI^{-1/2}$, becomes a direct measure of the distance between the two charged surfaces*. Chromatography provides an unique means of actually measuring this equilibrium distance and thus the extent to which a polyelectrolyte perturbs the surrounding solvent. To this end one notes that the diffuse double layers of each of the opposing surfaces superimpose additively where they overlap weakly[71]. The potential minimum reflects the pertinent level of thermal noise and may be taken as a reference point for an operational definition of the thickness of the double layer. The thickness is defined as the distance from the surface to a point where the potential is equal to the minimum of the overlapping potentials. The equilibrium distance then is slightly larger than the sum of the thickness of the two constituent double layers. Taking the elution data for the Mono-S column, which carries a saturating layer on the matrix, the equilibrium distance for ovalbumin is 4.0 times the Debye length and for MS2 is 6.8 times the Debye length, both corresponding to saturated layers on the macromolecule. A rough estimate of the maximum dimension of a double layer then is 1–2 times the Debye length around a spherical particle of radius 3 nm and 3 times the Debye length for spheres of radius 14 nm.

It would be of considerable interest to compare such figures with data obtained from scattering experiments[97,98] and electron microscopy (see discussion of inverse chromatography). An indication of the magnitude of the double layer is provided by the fact that force measurements between sheets of mica have been extended to distances beyond 5 times the Debye length (see ref. 99). In gels of tobacco mosaic virus (TMV), interparticle spacings as large as 8 times the Debye length have been measured and it has been estimated that the level of "thermal pressure" corresponds to 12 times the Debye length[98], which means that each double layer contributes 4–6 times the Debye length in the absence of applied pressure. At these distances, the Van der Waals attraction equals the electrostatic repulsion. Similar measurements have been conducted with muscle[98] and with DNA and membranes (see ref. 100). At short distances (< 1 nm) hydration forces become dominant[98–100] and may explain why the elution becomes independent of the ionic strength at high ionic strength.

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* Since $R = R_\eta + \kappa^{-1}x = R_\eta + 0.3I^{-1/2}x$ (in water at room temperature) it follows that $dR/dI^{-1/2}$ = $0.3x$, where x is the distance between opposing surfaces in multiples of the Debye length and reflects the electrostatic equilibrium distance. Inspection of eqn. 3 or 4 reveals that x must increase if a increases in order for Ψ to remain at the same level of thermal noise; x also depends on Ψ_s.

Rather surprisingly, even the "best" matrix (TSK 5000PW-3) exhibits half the max-imum effect which was observed for a saturated double layer around the matrix (Mono-S). Given the requirement to conserve electroneutrality as much as is com-patible with the formation of a diffuse double layer, the replacement of a counter ion by a macromolecule without charged sites is however also energetically unfavourable since it increases the charge separation between polyelectrolytes and counter ions. Thus the diffuse double layer should become impenetrable even for a perfectly neutral matrix which predicts effects of the observed magnitude. That electroneutrality is indeed conserved throughout a chromatographic column has been verified experi-mentally[66]. The generalized extension of this principle also provides a means for studying ligand binding[101].

Judged by the excellent correlation of electron microscopy with the hydrody-namic length of rods determined from SEC data at low ionic strength[73], the ionic strength dependence seems to be nearly independent of the particle shape within the errors of measurement. This is corroborated by a more recent study which reports a systematic variation of the ionic strength using dimers and tetramers of the chy-motryptic rod fragment of desmin whose $dR/dI^{-1/2}$ variation parallels those of glob-ular macromolecules of similar viscosity radii[102]. Thus, SEC may be employed in any solvent, including low ionic strengths, to determine the shape of macromolecules in solution (cf. refs. 10 and 103). In addition it provides information on colloidal properties that seem important for understanding biocatalysis, macromolecular assembly and numerous other biological and technological issues. Polycations, that adsorb to the weakly negatively charged regular SEC matrices in an anion-exchange mode, may equally be studied on anion-exchange resins of low surface charge density. Since IEC properties are suppressed at high ionic strength, elution patterns on op-positely charged matrices should become identical which may serve as a quality con-trol in determinations of viscosity radii.

In principle, SEC reflects both the dimensions of the polyelectrolyte and its diffuse double layer of counter ions. The latter may however be neglected at high ionic strengths where the compact double layer is much smaller than the size of the macromolecules. Similarly, the solvation layer around a macromolecule will be de-termined by preferential exclusion or attraction of all solution components present, which could lead to yet another difference between chromatographic and bulk values of R_η since SEC measures the total dimension whereas bulk experiments reflect only the slowly exchanging fraction of the total solvation. This conclusion is also relevant to SEC of non-electrolytes whose solvation may differ in different solvents, and are thus eluted differently even if the bulk intrinsic viscosity remains unchanged and no adsorption to the matrix takes place.

Ion-exchange chromatography

IEC is presumably a stepwise process of binding and dissociation from the matrix. The underlying forces are short range and are dominated by the replacement of tightly bound counter ions by the macromolecule[95]. This view is emphasized by the present observation that ion-exclusion effects on IEC and SEC columns are of similar magnitude, confirming that most of the counter ions of an IEC matrix are tightly bound to the matrix. The data further indicate that the retention is determined by local charge clusters for polyelectrolytes with near zero net charge (Fig. 3), but

increasing the net charge makes the latter a dominant factor under typical IEC conditions, *viz.*, one pH unit difference from the isoelectric point. Saturation effects are, however, equally present in IEC and have led to the concept of effective charge rather than net charge[104–106]. It will be interesting to explore the relationship between the net charge of titration, the effective charge of IEC, Ψ_s as measured by SEC, the surface potential measured by field-sensitive dyes and other probes[96,107], the electrokinetic potential (electrophoretic charge) and the charge effective in the Donnan terms of second virial coefficients[108,109]. Another thus far unaccounted factor of elution is the size of the macromolecule that may have a profound influence on the ionic strength dependency of IEC elution. The results in Fig. 5 in combination with the data equivalent to those in Fig. 4 show that large highly charged molecules may be eluted sooner from a salt gradient than a smaller less charged macromolecule, depending on the porosity of the matrix. Otherwise IEC is governed by principles entirely different from those of SEC.

Inverse chromatography

The mutual interaction of polyelectrolyte macromolecules with a polyelectrolyte microporous matrix has been investigated using well defined rigid gels. It has been demonstrated that information regarding the solution properties may be obtained by chromatographic procedures. However, the opposite is equally feasible, namely to study unknown gels and polymer networks, such as the cytoskeleton (the scaffolding proteins of cells), with known macromolecules. In this manner it will be possible to elucidate the inhomogeneity in the spatial distribution of polymers forming such networks and of the factors that govern cross-linking and bundling. Colloidal forces should play an important role not only for the mutual interaction between gel and probing macromolecule but also for the supramolecular organization itself. Inverse chromatography is particularly advantageous for studying soft gels in different degrees of swelling and organization; it is however promising for all porous

TABLE II

AQUEOUS SEC AND IEC COLUMNS

Column*	R_η exclusion** (nm)	Maximum pore radius*** (nm)	Average pore radius§ (nm)
TSK 6000PW	200	210	100
TSK 5000PW	55	65	36
Mono-S	45	54	31
Superose 6	25	29	25
TSK 4000SW	22	26	18
Superose 12	13	16	14
TSK 3000PW	10	13	6
Zorbax I-250	8.5	11	9

    * Data taken from ref. 10 and the present study.

    ** Defined by extrapolation of the log-linear regime of elution of polyelectrolytes to the void volume at high ionic strength.

    *** Polyelectrolyte exclusion size without the size of the double layers (rounded).

    § From $R_\eta + (dR/dI^{-1/2})I^{-1/2}$ at $V_{1/2} = (V_{\rm VOID} + V_{\rm TOT})/2$ multiplied by a geometry factor of 2.7.

materials. Polyelectrolytes of rigid structure seem to be ideal probes, ion-exclusion effects minimize adsorption and the electric double layer may now be properly taken into account and controlled. Neutral macromolecules are not only adsorbed to the matrix in most solvents[27], but are also likely to be solvated to different degrees, a critical factor for absolute pore-size measurements which was not recognized in the past. To attribute differences in the calibration graphs solely to different degrees of solvation of the matrix (see Introduction) seems oversimplified in the light of the present findings.

The average pore size effective in a separation depends on the surface-to-volume ratio and a geometric parameter[29,31]. A number of rigid matrices have been studied and it was estimated that the mean pore size is 2.7 times the size of a sample eluted at $V_{1/2} = (V_{VOID} + V_{TOT})/2$[31], but values ranging from 2.5 to 3.0, possibly up to 3.5, were observed[32]. By proper choice of reference dimensions for the probing macromolecules, reliable pore-size distributions should be generated. The average pore dimensions calculated in this manner for the matrices investigated are summarized in Table II. The ratio of the exclusion limit to the average pore size is a measure of the pore-size distribution and thus of selectivity.

These considerations suggest that inverse chromatography may be useful for studying the porous, electrostatic and structural properties of polymer networks and polyelectrolyte assemblies such as those in natural and synthetic membranes and cellular arrays made up of cytoskeletal proteins. Since chromatography has already proved to be valuable in guarding against preparation artifacts in the electron microscopy of dispersed macromolecules[10,73,110], it is hoped that extension of the present studies will make it possible to do the same for polyelectrolyte networks.

CONCLUSIONS

The mechanism of separation in SEC is based upon the unperturbed dimensions of the molecule which include the bulk viscosity radius, the double layer and other solvent interactions around the macromolecule. Elution of coiled polymers is further complicated by the fact that the bulk viscosity radii depend on coil expansion which itself varies with ionic strength and solvent. Molecules passing through the microporous matrix of column materials have planes of shear comparable to those in the bulk. Wall effects are the cause of important differences with respect to measurements of bulk hydrodynamic properties. It was found that hydration forces, differential solvation and in the case of polyelectrolytes the diffuse double layer prohibit approach to the wall over surprisingly large distances and thereby reduce the pore volume accessible to the solute. The role of the double layer was analyzed in detail and shown to increase in size with both the net charge up to a level of saturation and with the size of the macromolecule. The repulsion from the wall also increases with the charge density of the matrix up to a saturation level. Nonetheless, bulk viscosity radii may be calculated from SEC data and it was also shown how to obtain molecular weights and how to exploit the findings for preparative strategies. Both SEC and IEC columns can be used for SEC measurements. However there is a principal difference between the separation mechanisms of SEC and IEC. Molecular properties may be inferred either for unknown macromolecules using matrices of known structure or for unknown matrices with known polymers.

ACKNOWLEDGEMENTS

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