POLYELECTROLYTE CHARGE CORRECTED MOLECULAR WEIGHT AND EFFECTIVE CHARGE BY SEDIMENTATION

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ABSTRACT Ionic charge on a macromolecule complicates the determination of its molecular weight in solution due to the Donnan effect. Compensation for it can be made if one knows the value of the effective charge, which can be found by dialysis equilibrium across a semipermeable membrane. A moving boundary of molecules sedimenting in a centrifugal field can act as a membrane, obviating some of the disadvantages (such as selective adsorption) of a real membrane. Interference optics are used to monitor the reverse gradient of the salt due to the Donnan effect, hence facilitating the determination of the effective charge. The apparent molecular weight obtained from a conventional sedimentation equilibrium can then be corrected to yield the true molecular weight. The effective charge is valuable in revealing macromolecular structural features when related to the titratable charge through the Manning counter-ion condensation theory. Agreement between the values of the backbone molecular weights for the Na, Cs, and Ca salts of heparin indicated the validity of the approach. The effective charge ratio and the axial charge spacing for the Na and Ca heparin agreed with the literature, whereas the results for Cs indicated a degree of binding in excess of that due to counter-ion condensation.

INTRODUCTION

It is well known that the presence of electrical charge on a macromolecule complicates the determination of its molecular weight by physical methods. The molecular weight observed without taking certain precautions will be less than that expected, i.e., it will more closely approximate the equivalent weight. One may deal with this problem by either reducing the charge on the molecule by shifting the pH (if the molecule is zwitterionic), or naively, "swamping out the Donnan effect" by adding a large quantity of a salt that has one ion in common with the polyelectrolyte. Even though the addition of an excess of a third ionizable component causes ion shielding, thereby reducing the inter-macromolecular (long range) interaction, in the limit of zero polymer concentration at finite salt concentration there still remains the short range polymer–small ion interaction. Therefore, even in the presence of salt, unless precautions are taken the apparent molecular weight (at zero polyelectrolyte concentration) will be lower than the correct one. Williams et al. (1) derived an equation for the distribution of the two charged components (polyelectrolyte and a uni-univalent salt with an ion in common with the polyelectrolye) of such a three component system at sedimentation equilibrium. When it was simplified and solved in terms of the apparent molecular weight (Ma) of the polymer they obtained

$$Ma = \frac{Mp\left\{1 - \frac{Z}{2}\frac{Ms\theta s}{Mp\theta p}\right\}\left\{1 - \frac{Z}{2}\frac{Ms}{Mp}\frac{(1 - \bar{v}_s\rho)}{(1 - \bar{v}_p\rho)}\right\}}{\left\{1 + \frac{Z^2}{2}\frac{Ms}{Cs}\frac{Cp}{Mp}\right\}}. \quad (1)$$

Mp and Ms are the molecular weights of the polymer (including the counter-ion) and salt respectively; $\bar{v}$, θ, and C are the partial specific volume, the refractive increment, and the concentration (wt/vol) of the two components; ρ is the density of the solution, and Z is the absolute value of the effective charge on the polymer molecule.

This equation is limited to short column equilibrium sedimentation as it does not allow for the density change across the cell. In practice, short columns are used routinely to shorten the time to attain equilibrium. In addition one can use in a computer program Eq. 1 in conjunction with the equation for equilibrium sedimentation

$$C_r = C_0\rho^{\sigma(r^2/2 - r_b^2/2)}$$

where

$$\sigma = \frac{M(1 - \bar{v}\rho)w^2}{RT}, \quad (2)$$

(where w and r are the angular rotation and radial distance, respectively) to provide through an iterative process the density of the solution and the concentrations of both the salt and the polymer at each radial position. Eq. 1 has been somewhat simplified in that terms of the order $(Z \cdot Ms \cdot Cp)/(2 \cdot Mp \cdot Cs)$ are dropped to improve the intuitive understanding of the physical phenomena. This simplification has a negligible effect on the results if the ratio of salt to polyelectrolyte concentration is large or the charge is not too high. Any experimental study, however, should eventually consider carefully the effect of this omission on final results.

Using the same derivation procedure as Williams et al. (1) one can show that the general form of Eq. 1 is

$$Ma = \frac{\left\{1 - \frac{Z}{w(w+y)}\frac{Ms\theta s}{Mp\theta p}\right\}\left\{1 - \frac{Z}{w(w+y)}\frac{Ms}{Mp}\frac{(1-\bar{v}_s\rho)}{(1-\bar{v}_p\rho)}\right\}}{\left\{1 + \frac{Z^2}{wy(w+y)}\frac{CpMs}{MpCs}\right\}}, \quad (3)$$

where w and y are the absolute values of the charge on the counter-ion and co-ion of the polyelectrolyte, respectively, and terms $ZMsCp/[(w + y)MpCs]$ are dropped as being negligible. Since the two parenthetical terms in the numerator are each less than 1, it is clear that Ma will always be less than Mp no matter how great the ratio of salt to polymer concentration. As the ratio is increased, the value of the denominator approaches one. Rewriting Eq. 1 or Eq. 3 as Cp goes to zero at finite Cs,

$$Ma_0 = Mp \cdot (A) \cdot (B)$$

or

$$Mp = Ma_0 \cdot K, \quad (4)$$

where A and B are the two parenthetical terms in the numerator of Eq. 1, K is the reciprocal of the product of A and B, and Ma_0 is Ma at vanishing concentrations of polymer. Term A arises because of the Donnan effect (reflected in the term $ZMs/[w(w + y)Mp]$ and the use of refractometric optics which measures the combined concentration of the salt and the polymer in the ultracentrifuge cell. It can be eliminated by using a technique that measures the concentration of the polymer alone, such as absorption spectrophotometry. Since the values of A and B are approximately the same, this has the effect of reducing the correction term (K) to the square root of its maximum size. The B term arises as a result of a combination of the Donnan effect, the distribution of the salt in the centrifugal field, and the electrical interaction between the counter-ions and the polymer backbone charges. This term times Mp represents the value of the molecular weight of the Scatchard definition (2) of component 2 (i.e., $Mp - ZMs/[w(w + y)]$. The value of the term can be made to approach the value of 1 by the use of a salt that has a partial specific volume with a value close to one. Braswell and Lary (3) successfully used such a neutral density supporting electrolyte (triethylamine hydrochloride) in a study of associating cationic dyes. The correction factor (K) for a material such as Na-heparin, which has no available absorption band, so that refractometric optics must be used, and having Mp = 20,000 and Z = 200 (assuming that every titratable charge is "effective", i.e., contributes to the Donnan effect), can be as large as 1.7. Therefore, to accurately determine the molecular weight of many polyelectrolytes using this approach, the effective charge must be known, so that the value of K can be calculated to correct Ma_0.

Braswell (4) rewrote Eq. 1 (for the case of NaCl) in the form of a linear equation, where the reciprocal of the apparent molecular weight is expressed as a function of Cp, as follows:

$$1/Ma = [1/(Mp \cdot A \cdot B)] + [(Z^2 \cdot Ms)/(2 \cdot Mp^2 \cdot A \cdot B \cdot Cs)] \cdot Cp. \quad (5)$$

The slope divided by the intercept of this equation (Si) is seen to be twice the value of the second virial coefficient for a charged molecule (reference 5). Since Eq. 1 was derived assuming that the only nonideality present is that due to charge, a term for the excluded volume virial coefficient is not present. Upon writing the expression for the result of dividing the slope by the intercept (Si) and solving for Mp we get

$$Mp = (Z^2 \cdot Ms)/(2 \cdot Cs \cdot Si). \quad (6)$$

Substituting the value of Mp found in Eq. 6 into Eq. 1 and realizing that at Cp = 0 the denominator is equal to one, and Ma is equal to Ma_0, one obtains a quadratic equation in Z. From this equation and the experimentally determined values of Ma_0 and Si, both obtained from a graph of $1/Ma$ vs. Cp (Eq. 5) Braswell (4) found Z and thus (from Eq. 6) Mp for a number of heparin samples. The assumption that the excluded volume nonideality is minimal was considered reasonable for molecules of this weight (Mp = 20,000 D). The values reported for the equivalent weights of the unaltered heparin samples were in the range 443–810. Since the equivalent weight calculated from the titration of acid groups is from 180 to 190 it is obvious that the slopes of plots of $1/Ma$ vs. Cp is less for heparin than one would expect. This could mean either that only a fraction of the charges are "effective" or that there is a considerable negative contribution by the excluded volume virial coefficient or some combination of the two factors.

Manning has shown in a series of papers (for an excellent review see reference 6) that if the charge density z/l (where l is the linear distance over which the titratable charge z is spaced along the cylindrical axis of the molecule) is above a critical value, a number of counter-ions will "condense" onto the charges rendering them neutral. This continues until the charge density is reduced to the critical value, thus reducing the effective charge. Therefore, highly charged polyelectrolytes should be expected to have an effective charge fraction (i.e., Z/z) of less than one. If the effective charge could be determined independently one could obtain not only the correct molecular weight (Mp) from sedimentation equilibrium studies, but could also use the slope method to determine the excluded volume virial coefficient.

In an alternate approach to the determination of the molecular weight of polyelectrolytes Casassa and Eisenberg (7–9) (see review by Eisenberg [10]) described an

experimental procedure which bypassed the need for knowing the effective charge. Dialysis of a series of concentrations of the polyelectrolyte against a quantity of solvent containing an exact concentration of salt so that the diffusible components are at constant chemical potential is followed by the determination of the apparent partial specific volume (ϕ') of the polyelectrolyte from the densities of the dialyzed polyelectrolyte solution using the density of the solvent as the reference density. It is obvious that such a determined partial specific volume will be different from that obtained in the standard manner ($\bar{v}$), because the concentration of salt present in the dialyzed polyelectrolyte will vary with the concentration of polyelectrolyte due to the Donnan effect. Casassa and Eisenberg then showed that in the limit $Cp \Longrightarrow 0$

$$\left(\frac{\mathrm{d} \ln Cp}{\mathrm{d}r^2}\right)\frac{2RT}{w^2} = (1 - \phi'\rho^\circ)Mp = (1 - \bar{v}_p\rho^\circ)Ma \qquad (7)$$

where ρ° is the density of the solvent. Therefore by determining the value of ϕ', one can unambiguously determine the correct molecular weight from equilibrium sedimentation without explicitly knowing the effective charge. They also showed that

$$Ma = Mp \cdot \Lambda$$

where

$$\Lambda = 1 + \xi(1 - \bar{v}_s\rho^\circ)/(1 - \bar{v}_p\rho^\circ). \qquad (8)$$

It can be seen that when the B term in Eq. 1 is written as

$$Ma = Mp \cdot [1 - (Z \cdot Ms/2 \cdot Mp)] \cdot (1 - \bar{v}_s\rho^\circ)/(1 - v_p\rho). \qquad (9)$$

that

$$\xi = -(Z \cdot Ms/2 \cdot Mp). \qquad (10)$$

The term ξ can be considered a binding term for the salt to the polyelectrolyte and is negative because after dialysis is complete, there is a higher salt concentration in the compartment containing only salt than there is in the polymer containing one.

If one were to take data from the described dialysis experiments differently, i.e., as salt concentrations on both sides of the membrane, one could calculate the effective charge on the polyelectrolyte molecule (at that concentration of polyelectrolyte) by the Donnan equation. Then Eq. 1 can be used (remembering that term A is only evoked if one cannot determine the concentration gradient of the polymer alone) to determine the correct value of the molecular weight of the polyelectrolyte bypassing the need for explicitly determining the value of ϕ'. It is therefore apparent that there is great similarity between the Williams et al. and the Casassa–Eisenberg approaches.

In still another method, Edelstein and Schachman (11) and Thomas and Edelstein (12) (reviewed by Edelstein and Schachman (13) evaluated simultaneously the values of both the apparent partial volume (ϕ') and the molecular weight by equilibrium sedimentation, first in light, and second, in heavy water. This approach has the disadvantage of containing the assumption that the preferential interaction coefficients have the same values in the different solvents. It is also not as accurate as the other methods unless the heavier (and expensive) D_2O^{18} is used.

Since the basis of the effect of charge on molecular weight is the Donnan effect, the obvious way of measuring effective charge is by dialysis equilibrium. The dialysis experiment is set up in the common manner, i.e., a given weight of polyelectrolyte is dissolved in a definite amount of an aqueous salt solution (Vp) of molal concentration ms to make a polyelectrolyte solution of molal charge concentration of mp which is then dialyzed against a definite volume of solvent (Vs). At equilibrium, the resulting concentrations (ignoring activity coefficients) will be (for the Na salt of an acidic polymer in NaCl for example)

$$\frac{mp + ms - x/Vp}{ms + x/Vs} = \frac{ms + x/Vs}{ms - x/Vp}, \qquad (11)$$

where x represents the number of moles of Na^+ or Cl^- which have either moved into or out of the compartment. Eq. 11 will be recognized as a common form of the Donnan equation. The term on the left represents the equilibrium concentrations of Na^+ ions in the polymer compartment (numerator) and the solvent compartment (denominator), whereas the right term represents the equilibrium concentrations of the chloride ion in the solvent containing compartment (numerator) and the polymer compartment (denominator). Upon cross multiplying one obtains a quadratic in x. Solving for Δ, which is defined as being the difference in the salt concentrations between the compartments (i.e., $\Delta = x/Vs + x/Vp$), for the case where $ms >> mp$ (which causes the ratio of the volumes to become unimportant) yields

$$\Delta = mp/2 = CpZ/2Mp. \qquad (12)$$

Measuring the salt concentration difference between the inner and outer compartments will therefore provide one with the effective charge. The best way of doing this is by a differential method such as differential refractometry or interference. However, the concentration of the polymer must be determined independently so that its contribution to the refractivity can be calculated.

When a solution of a polymer is centrifuged at high speed so that a boundary is formed, one effectively produces a dialysis cell without a membrane. This should be advantageous as it would eliminate problems of adsorption or elution of materials into or out of the membrane and also obviates the need for density measurements while providing values for the effective charge. Such a technique would undoubtedly have value in other types of dialysis equilibria, such as binding studies. Therefore, with these

goals in mind, a study of the Donnan equilibrium during sedimentation was initiated. The rationale of this investigation is: measurement of the *Ma* of three salts (Na, Cs, and Ca) of the same polyelectrolyte followed by determination of the effective charge to correct the value of *Ma*, followed by deduction of the weight of the counter-ions should lead to identical values for the molecular weight of the macromolecular backbone if the technique is valid.

EXPERIMENTAL

General

Heparin was used for the study because of its high charge, its availability, and also because the charge density is known. Since it is necessary to clear the meniscus region of polyelectrolyte to make a proper measurement, heparin ($s = 2$) is about the smallest molecule possible to study in this way. Therefore, the ultracentrifuge was run at close to maximum speed for these studies. The sodium heparin used was a nonfractionated commercial lung heparin (lot No. 13127, Organon Diagnostics, West Orange, NJ) of 134 U/mg anticoagulant activity. 1-g portions were dissolved in 25 ml each of 1 M NaCl, CsCl, and $CaCl_2$. The three solutions were then dialyzed against 500 ml of its respective salt at 1 M concentration. The dialysate was changed after 24 h and the dialysis was continued for an additional 24 h. The heparin solutions were then dialyzed against distilled water for several days, changing the dialysate every day, after which the samples were freeze dried. A 100-mg portion of each heparin was then dried over P_2O_5 in vacuo at 80°C in an Abdehalde drying pistol. This procedure was shown by us in an earlier report (4) to completely dry heparin without affecting its anticoagulant activity. The thoroughly dried samples were then dissolved in their respective salt solutions (concentration of salt to make an ionic strength of 0.15) to make 1% heparin stock solutions. Partial specific volumes were determined on the stock and dilutions of the stock solutions using the appropriate salt solution as the diluent, by means of a density meter (model DMA-02D, Mettler Instrument Corp., Hightstown, NJ). The values obtained are: 0.38 for Na, 0.32 for Cs, and 0.35 ml/g for Ca heparin. Similar measurements on the solvents yielded 0.30 for Na, 0.25 for Cs, and 0.18 ml/g for Ca chloride. Differential refractive index measurements were made on the heparin solutions (vs. the appropriate salt solution), and on the salt solutions (vs. distilled water), using a Phoenix differential refractometer. The values found are 0.134 for Na, 0.095 for Cs, and 0.144 ml/g for Ca heparin. The values for the respective salt solutions are 0.172, 0.080, and 0.238 ml/g. The titratable charge equivalent weight (Mp/z) was determined on the Na heparin sample by dye binding (4) as 180 g/mol of charge.

Equilibrium Sedimentation Studies

The molecular weights of the three heparins were determined by equilibrium sedimentation by means of Rayleigh interference optics. For each heparin type, three to five different concentrations were studied ranging from 0.1 to 0.3% heparin. Double sector cells (30-mm long) were filled so that the solution column was ~3-mm high. The solvent side was filled slightly higher with the appropriate salt solution. The centrifuge was run at 9,000 rpm for 24 h, at which time it was found that equilibrium was essentially attained. The interference pictures were read on an automated plate reader developed by Laue and Yphantis (14, 15) and the fringe count across the cell determined. The Lansing–Kraemer (16) method was used to calculate the over-all-the-cell *Ma* at a given heparin concentration. For this method one usually performs a synthetic boundary cell experiment on a portion of the sample to determine the fringe count corresponding to the initial concentration. Because the Donnan effect causes a progressive decrease in the fringe count with time during the run, an alternate procedure involving measurement of the difference between the refractive index of the solution and the solvent followed by calculation of the fringe value of this difference was performed before the experiment. The reciprocal of the values of *Ma* obtained from the Lansing–Kraemer calculation were extrapolated to zero concentration by a least squares program from which the intercept (Ma_o) and the slope were determined with standard deviations of 3–4%.

Velocity Sedimentation Studies

Fig. 1 shows (in an idealized manner, in that the salt gradient produced by sedimentation is not shown) the principles upon which the determination of effective charge by dialysis equilibrium during a velocity sedimentation run is based. The Donnan effect will cause salt to be pumped across the boundary, upward toward the meniscus. Therefore, in the boundary region one will have a positive gradient due to the polyelectrolyte and a smaller negative one due to the salt. The Rayleigh interference system on the ultracentrifuge can be used to measure the change in refractive index radially down the cell. The change in refractive index observed in moving from the region above the boundary to the plateau region is due to the concentration of the polyelectrolyte in the plateau minus that due to the concentration difference of the salt across the boundary. The polyelectrolyte concentration at the plateau (expressed conveniently in fringes) at a particular time during a run (Cp'), is equal to that measured before the experiment by differential refractometry against the solvent (Cp) reduced by the amount of radial dilution that has occurred by that time. Subtraction of the refractive index change across the boundary during an experiment from the corrected concentration of polyelectrolyte yields the increase of salt concentration (Δ) in the region between the boundary and the meniscus. Using this ideal picture, at very early times when the volume of this region is small, if the meniscus concentration of polyelectrolyte is zero (due to the application of an almost instantaneous extremely strong centrifugal field), the dialysis will be at equilibrium. Under real conditions and at later times when one is actually obtaining data, the volume above the meniscus will be relatively large and increasing in size, and the conditions for dialysis equilibrium will not be obtained. Therefore to arrive at the proper equilibrium value of the salt concentration, it is necessary to extrapolate the value of $Cp' - \Delta$ measured at various times back to zero time. An example of one such extrapolation is shown in Fig. 2. As can be seen in the figure, data taken at times so early that the meniscus concentration of polyelectrolyte has not yet dropped to zero must be ignored. To reduce the time necessary to accomplish this, the highest practical centrifuge speeds are used.

In practice interferograms were first made of the cell with solvent in both channels to be later used as a "blank" to account for any small degree of optical variation in the cell. The cell was then thoroughly cleaned and dried in a vacuum desiccator without disassembling the cell before filling with solvent and sample. The measurement of the interference images on the plates were performed on the Laue–Yphantis automated plate reader (14, 15), which made it possible to read refractive index differences to one-hundredth of a fringe with a precision of about two-hundredths of a fringe. Several hundred points could be taken from a single picture in under one-half hour. This kind of sensitivity, precision, and convenience are essential for one to attain reasonable results, as the quantities of salt pumped produce changes in refractive index of less than a fringe. Attempts to read the plates manually on a Nikon Shadowgraph comparator yielded poor precision with much onerous work.

As shown earlier the determination of Δ, the difference in salt concentration due to the Donnan effect in a velocity sedimentation experiment, makes possible the calculation of the effective charge equivalent weight (Mp/Z). It can easily be shown from Eq. 11 that in an equilibrium dialysis of a polyelectrolyte with a univalent counter-ion, which is common to the uni-univalent salt against which it is dialyzed, for the case where the volume in which the polymer resides is much greater than that containing only the salt (the present experimental situation) that

$$mp = (\Delta^2 + 2 \cdot \Delta \cdot ms)/ms, \qquad (13)$$

where mp and ms are the molal equivalent concentrations of the polymer and salt, respectively. Since at low concentrations of polymer mp =

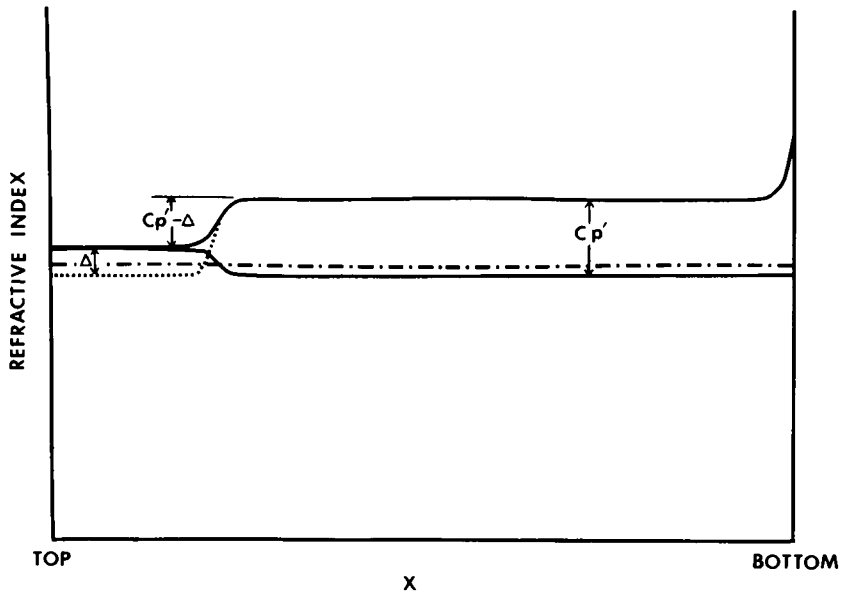

FIGURE 1 This schematic representation of equilibrium dialysis occurring simultaneously with the velocity sedimentation of a polyelectrolyte in the presence of a salt shows the apparent decrease in the refractive index of the polyelectrolyte (Δ). This decrease is produced by the salt gradient (shown as the solid line which decreases going to the right) due to the Donnan effect, which is opposite to the gradient of the polyelectrolyte (shown as the dotted line which increases going to the right) produced by the centrifugal field. The resultant is shown as the solid line which increases going to the right. The diagram is idealized in that the salt redistribution is shown as being at Donnan equilibriuim and not affected by sedimentation. Cp', the plateau concentration of polyelectrolyte is the initial concentration of polyelectrolyte Cp, reduced by radial dilution. The quantity $Cp' - \Delta$ is directly measured during the experiment as a fringe count difference, whereas the quantity $Cp - \Delta$ is the value obtained when these experimental values are extrapolated to zero time (see Fig. 2). The dashed line represents the original salt concentration.

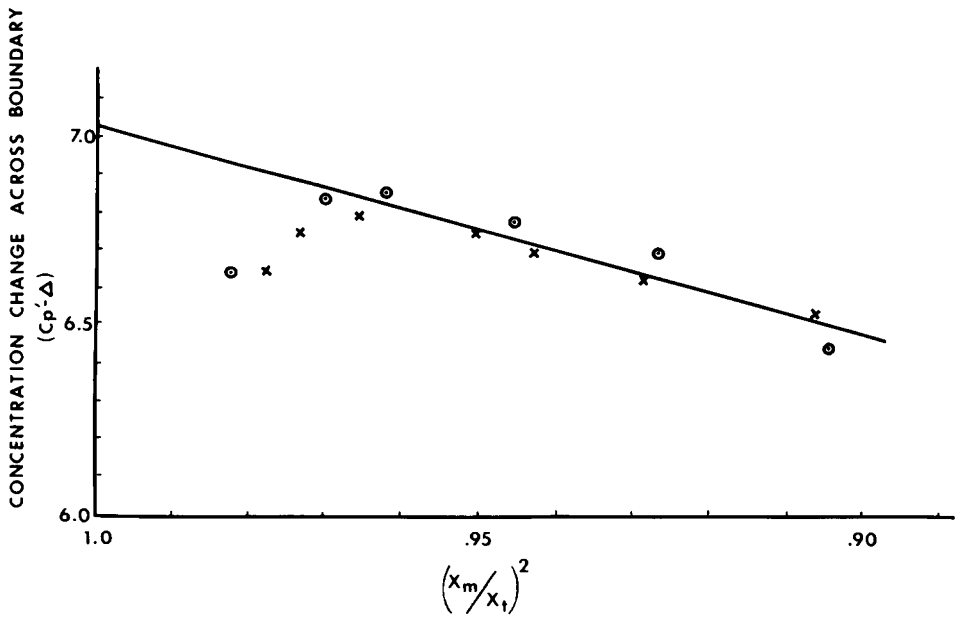

FIGURE 2 Example of data from two sedimentation experiments. Na heparin (Cp = 0.267% or 7.86 fringes in a 1.2-cm cell) in 0.15 M NaCl centrifuged at 64,000 rpm. Extrapolation to zero time of only those data points ($Cp' - \Delta$) obtained after the meniscus concentration of polyelectrolyte had reached zero (in this case the points forming the line with the negative slope), yields $Cp - \Delta$. Previous knowledge of Cp from a refractive index measurement before the experiment therefore yields the value of Δ. Xm is the position of the meniscus, Xt is the position of the maximum gradient of the polyelectrolyte at time t, therefore Xm/Xt is a measure of time since commencement of sedimentation. Squaring this term linearizes the graph and corrects it for radial dilution. The zero time extrapolation equals 7.02 ± 0.05 fringes, therefore Δ = 0.84 ± 0.05 fringes yielding an equivalent weight of 346 ± 23 dalton/charge. Upon correction for the effect of unmatched menisci and therefore salt gradients, Δ becomes 0.74 ± 0.05 fringes yielding an equivalent weight of 393 ± 23.

$Z \cdot Cp/Mp$, if $ms >> mp$ then $mp = 2\Delta$, as shown before for the general case for any ratio of compartment volumes. Since the ratio of salt to polymer charge concentration is not always great however, it is generally necessary to use Eq. 13. Therefore a knowledge of Δ, Cp, and Cs (or ms) allows one to determine the equivalent weight of the polyelectrolyte. Substitution in Eq. 1 of the value of Ma_0 and Mp/Z yields the value of Mp. For the case of a bivalent counter-ion (Ca of Ca-heparin) in common with a bi-univalent salt (such as $CaCl_2$) the Donnan equation (Eq. 11) must be set up somewhat differently. As a result the bi-univalent analogy of Eq. 13 becomes

$$mp = (\Delta^2 + 3 \cdot \Delta^2 \cdot ms + 3 \cdot \Delta \cdot ms^2)/ms^2. \quad (14)$$

In the event that $ms >> mp$ then $mp = 3\Delta$. Since the calcium salt of heparin was studied in the presence of $CaCl_2$ it is necessary to rewrite Eq. 3 for that salt. Upon substituting the values of 2 and 1 for w and y, respectively, one obtains an equation similar to that for NaCl (Eq. 1) except that every number 2 is replaced by the number 6.

It is common practice when performing ultracentrifuge experiments to allow the amount of solvent to be slightly greater than the amount of solution, so that there is a small mismatch in the positions of the menisci. This is to assure that none of the data is lost due to the solvent meniscus being lower than that of the solution. It was found that the results of the Donnan study were profoundly affected by the degree of mismatch. A mismatch of 0.1 mm, for example, for the sodium heparin versus sodium chloride at 64,000 rpm led to an error of ~0.1 fringe which increased to ~0.3 fringe when cesium heparin versus cesium chloride was studied. It was found to increase linearly with increasing mismatch and increased with the square of the speed. The error arises from the large salt gradient produced at the menisci of both the salt and solution columns. If the menisci are not matched the salt gradients are not completely mutually cancelled. That the magnitude of the error is a large fraction of Δ, can be seen using the results shown in Fig. 2. The correction necessary was found to be 0.1 fringe leading to a true value of Δ of 0.74 yielding an equivalent weight of 393 instead of 346. To minimize the degree of mismatch without incurring the risk of having too little solvent, it was necessary to modify the cell. A small groove was made between the two cell channels at both the bottom and the top of the channels. Then after the cell was assembled, a small amount (0.1–0.2 ml) of a nonreactive silicone oil (DC 550, $\rho = 1.10$) was put into both cell channels before adding equal quantities of solvent and solution to their respective channels. With careful handling, mixing of solvent and solution does not occur, due mainly to the smallness of the groove and the layer of oil. Under centrifugation the menisci will move to reduce the differences between them, i.e., readjusting their heights by moving oil through the connecting groove at the bottom of the channels. The second groove facilitates air pressure equalization between the two channels. Several requirements must be met to optimize the matching. First, the density of the oil should be denser than the solution (and therefore the solvent) so that it will stay on the bottom. Second, the densities of all three liquids should be as similar as possible so that the system will be sensitive to differences in column height. The fact that the solution is denser than the solvent however, assures that the solvent column will always be slightly higher than that of the solution, by an amount that can be calculated. This results in a small error that can be corrected for by performing calibration experiments in unaltered cells with varying differences in column height.

There are several other ways of combating the problem of mismatching menisci. For example, we have recently become aware of a report by Ende (17) in which he describes a cell design (design No. 3) that causes the menisci of solvent and solution to be brought into alignment. It would appear that such cells would be ideal for these studies. Using another strategy, one could use a salt for which the value of $(1 - v\rho) \simeq 0$ (3, 18, 19). As a result there would be no salt gradient due to sedimentation and therefore no error due to improper leveling of the menisci (in addition to, as mentioned before, the elimination of term B in Eq. 1 or 3). With larger macromolecules, lower sedimentation velocities can be used to clear the meniscus of polyelectrolyte, thus reducing the degree of sedimentation of the salt, thereby reducing the need for such precautions.

The velocities for this series of experiments varied from 48,000–56,000 rpm for the cesium heparin to 56,000–64,000 rpm for the Ca and Na heparins. The results, in the form of effective equivalent weight (Mp/Z), from each velocity run were plotted against the polyelectrolyte concentration at which it was run. Within experimental error, over the concentration range studied, the results showed little concentration dependence (see for example Fig. 3).

RESULTS AND DISCUSSION

Because of the decrease in accuracy of the method with polyelectrolyte dilution and the absence of a significant dependency of the value of the equivalent weight on the polyelectrolyte concentration, the results shown in Table I were all taken at approximately the same high heparin concentration (i.e., 0.25–0.28%) where the accuracy was best. The first line of the table shows the effective equivalent weights (Mp/Z) determined for each of the heparin salts. A membrane dialysis experiment with Na heparin yielded a value of $3.8 \times 10^2 \pm 0.6$ for comparison with the value of $3.9 \times 10^2 \pm 0.2$ shown in the table. The formal equivalent weight (Mp/z) for Cs and Ca were calculated from that determined by dye binding for the Na salt (180 g/mol of charge) by adding the equivalent weight of each counter-ion from which is subtracted that of Na (i.e., for Ca, 20 minus 23). The charge corrected values of the

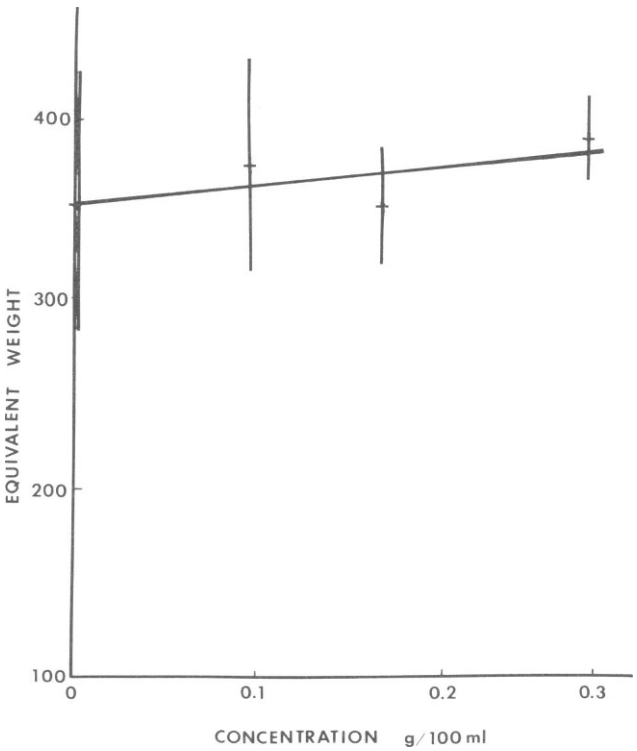

FIGURE 3 The equivalent weight of Na heparin as determined by Donnan equilibrium during a sedimentation velocity experiment. The error bars for the data points result from the errors in fitting curves similar to those in Fig. 2. The error calculated for the intercept of this graph is large enough to render the apparent slope of the data insignificant. The error in the equivalent weight increases with dilution because the size of the measurement error (0.03–0.05 fringes) is relatively constant while the measured value of Δ decreases with concentration.

TABLE I
RESULTS OF DONNAN EQUILIBRIUM STUDY OF HEPARIN WITH VARIOUS COUNTER-IONS (IONIC STRENGTH = 0.15) BY VELOCITY AND EQUILIBRIUM SEDIMENTATION

	Na	*Cs*	*Ca*
Mp/Z ($\times 10^{-2}$)	3.9 ± 0.2	9.3 ± 0.9	7.6 ± 0.7
Mp/z ($\times 10^{-2}$)	1.80 ± 0.05	2.9 ± 0.1	1.80 ± 0.05
Z/z	0.46 ± 0.04	0.31 ± 0.03	0.23 ± 0.02
Mp ($\times 10^{-3}$)	16.3 ± 0.7	26 ± 1	15.6 ± 0.7
Mb ($\times 10^{-3}$)	14.2 ± 0.9	14.1 ± 0.9	13.8 ± 0.9
Bt ($\times 10^{6}$)	4.3 ± 0.2	1.2 ± 0.1	0.30 ± 0.04
Bz ($\times 10^{6}$)	11.0 ± 0.6	1.9 ± 0.2	2.4 ± 0.2
Bv ($\times 10^{6}$)	−7 ± 1	−0.7 ± 0.3	−2.1 ± 0.2

Units of B are in mol · liter · g^{-2}.

molecular weight (Mp) are shown in the fourth line of the table. That the difference in the values of Mp are due to the equivalent weights of the counter-ions can be seen by the agreement of the values of the molecular weight of the heparin "back-bone," which were calculated by subtracting the weight of the counter-ions from Mp. That is,

$$Mb = Mp - z \cdot Ec \tag{15}$$

where Mb and Ec are the "back-bone" molecular weight and counter-ion equivalent weight, respectively. The excellent agreement between the values of Mb indicates the validity of the technique, especially when one considers the differences in the atomic weights and charges of the counter-ions studied. The effective charge fraction (actual charge/formal charge), shown in line 3 of the table, is the ratio of the equivalent weight determined by dye binding divided by the equivalent weight determined by dialysis equilibrium using sedimentation velocity. The Manning theory would interpret this to be the fraction of the charge not "condensed," but available in the ionic atmosphere of the polyelectrolyte. These values for heparin can be compared with those determined by small ion tracer diffusion studies by Ander (20) on the Na and Ca salts of another heparin sample (of unknown origin) of 0.43 and 0.18, respectively. According to the Manning theory (6) for a polyelectrolyte with a fixed average charge axial spacing, the effective charge fraction for a doubly charged counter-ion should be half that of the singly charged ion. The Ca and Na results here agree with this picture. The effective charge fraction resulting from the cesium counter-ion is somewhat low. In the absence of any other factor the Manning theory would predict that either the charge spacing is less for the Cs heparin, implying some contraction of the molecule, compared with that of Na and Ca heparin, or that there is present a certain degree of specific binding for Cs ions. According to Manning's theory (6) the spacing of the charged groups on the molecule can be expressed as

$$Sp = 7.14 \cdot n \cdot Z/z, \tag{16}$$

where Sp is the spacing (Å), n is the valence of the charge, and the constant is the spacing (Å) allowed for single charges that would result in a charge fraction (Z/z) of 1 (i.e., the condition under which there is no charge condensation). Using the values obtained for Na and Ca, the charge spacing is 3.3 Å, while the results from the Cs studies indicate a spacing of 2.2 Å (all with an uncertainty of about ±10%). The results of Ander (20) using Na and Ca heparin cited earlier yield a spacing of 3.1 and 2.6 Å (error unknown). The alternate explanation suggests, assuming no charge in axial charge spacing, that about one-third of the Cs is specifically bound. At present, we cannot say which hypothesis is true, but feel that the latter is probably more likely.

As stated in the introduction, the slope of a plot of $1/Ma$ vs. Cp yields the second virial coefficient, which for a small polyelectrolyte molecule should be largely due to charge. However, the slopes found for the heparins in the earlier work (4) are too small, considering the magnitude of the formal charge. Therefore, either the effective charge is much less than the formal charge, or there is a large negative contribution from the excluded volume or some combination of the two factors. Now that it is clear from this study that the effective charge is smaller than the formal charge for heparin it is of interest to calculate how much of the observed virial coefficient is due to excluded volume. Assuming that the observed second virial coefficient (Bt) is made up of two components; one due to charge (Bz) and one due to excluded volume (Bv) one can write

$$Bt = Bz + Bv. \tag{17}$$

The value of Bt is determined from the slope of a graph of $1/Ma$ vs. Cp. The value of Bz is calculated from the effective charge divided by the molecular weight (the reciprocal of the equivalent weight, see Table I) using Eq. 6 for NaCl or by performing the identical operation on Eq. 3, one can get the more general form, i.e.,

$$Bz = Z^2 \cdot Ms/[2w(w + y)Mp^2 \cdot Cs]. \tag{18}$$

Finally, the value of Bv is calculated as the difference between Bt and Bz. These values determined for the various heparins can be found in the table. The negative values of Bv suggest that there would be a tendency for heparin molecules to associate if there were no charge present, since a positive value of $\sim 7 \times 10^{-6}$ (mol · liter · g^{-2}) would be expected due to the excluded volume of a neutral flexible molecule of this extension. It is of interest to compare the results of this study with that found for an affinity fractionated highly active fraction (731 U/mg) of Na-heparin (22). In that study the dialysis was done conventionally and resulted in a value of Z/Mp of 430 for a value of Mp of 22,000. These results lead to a value of Bz of 9×10^{-6}, while the value of Bt determined from the slope of the plot of $1/Ma$ vs. Cp is 4×10^{-6}, thus leaving the contribution of Bv to be $\sim -5 \times 10^{-6}$. All these values (except for the higher molecular weight of the fractionated

heparin) are similar to those shown in the table for unfractionated Na heparin of a different origin.

The presence of polydispersity should be considered since we showed (22) that even an affinity fractionated highly active fraction of heparin has a value of Mz/Mw of 1.25 while that of unfractionated heparins have values between 1.5 and 2 (4). Extending the derivation of Eq. 3 to the case where there is molecular polydispersity but the equivalent weight stays constant (i.e., $M_i/Z_i = Eq$), one finds that Eq. 3 properly represents the weight average molecular weight (Mw) and the weight average charge (Zw). Since it can easily be shown that

$$Mw/Zw = Mn/Zn = Eq, \tag{19}$$

the equations yield the correct equivalent weight in the presence of molecular heterogeneity.

Finally, we must consider the terms of the type $ZCpMs/[(w + y)MpCs]$ that have been omitted. Substituting the values found for the various terms yields a total value of ~0.02 for the dropped term for all the salts studied. Since this means that the values of the molecular weights and equivalent weights will be in error by ~2% and since this is not greater than our standard deviation it seems that we are justified in the omission of these terms.

The technique developed in this study is equivalent to the Casassa–Eisenberg treatment except that the experimental complications of membrane dialysis are avoided, as is also the need to make tedious density measurements. Further, the additional information obtained regarding the effective charge at each polyelectrolyte concentration when used with the apparent molecular weight at that concentration and a Williams et al. type equation (Eqs. 1 and 3), helps to throw light on the non-charge related virial coefficient.

The technique is also similar to the Yphantis–Roark approach (21) in which the Scatchard (2) definition of components is used. Their elegant thermodynamic treatment is similar to that of Williams et al. (1) and Braswell (4) in that the charge is found from the slope of the $1/Ma$ data vs. Cp, thus assuming that the excluded volume (non-charge related) virial coefficient is negligible. The important point is that a dialysis equilibrium experiment is necessary to extract both the charge and the excluded volume virial coefficient.

The technique is convenient and rapid and requires only that a velocity and an equilibrium sedimentation run be performed; both of which can be done in the same cell with a single loading of sample, although usually a lower column height may be desirable for the molecular weight determination to reduce the time to equilibrium. In addition, the method is relatively accurate, and uses small quantities of material. For molecules with sedimentation coefficients in excess of 10, it will be especially convenient and accurate, because the velocities necessary to clear the meniscus and therefore the care necessary to match menisci will not be as great. It should therefore be an especially useful tool for studying the charge, and axial charge spacing, hence yielding information on the structures of various polyelectrolytes. This laboratory is currently investigating DNA structures in solution and extending the technique to the study of the binding of more complicated counter-ions and the binding of ligands in general.

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