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THE BINDING OF DIVALENT METAL IONS TO POLYELECTROLYTES IN MIXED COUNTERION SYSTEMS.

I. THE DYE SPECTROPHOTOMETRIC METHOD

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The dye spectrophotometric method for the measurement of the activity of divalent metal ions in polyelectrolyte solutions containing added electrolytes is discussed. The method is applied to mixtures containing the dextransulfate polyanion, NaCl, and $MgCl_2$ or $CaCl_2$. A two wavelength ratio method as applied to polyelectrolyte solutions is compared to the standard method which makes use of the previous determination of the dye-metal ion formation constant. The ratio method is found to be a convenient and reliable method which is not influenced by decomposition of the dye or by statistical errors in the extrapolation procedure. The activity coefficients as determined by the two wavelength dye spectrophotometric method are compared to results of Donnan exclusion measurements, and of EMF measurements using a calcium ion selective electrode. The results of the spectrophotometric method are equal to those of the two other methods within the limits of error in the latter. The spectrophotometric measurements can extend to much lower ion activaties than the other two methods, and can be done in the presence of a large excess of added electrolyte, yielding results of considerably improved precision when compared to Donnan and EMF methods.

1. Introduction

The measurement of the divalent metal ion activity or the free divalent metal ion concentration in polyelectrolyte solutions containing both uni-univalent and di-univalent electrolytes is of considerable practical interest. The binding of multivalent metal ions by polyanions including acidic polysaccharides, polypeptides, and polynucleotides is strongly dependent on the ionic strength of the added 1 : 1 electrolyte. This ionic strength dependence has recently been examined in detail by Manning [1] and Iwasa [2], who considered the specific polyelectrolyte characteristics of the binding process. These theories are fully predictive in that they describe the extent of binding in terms of a single polyion property, i.e. the average linear charge separation on the polymer backbone. The treatment of Manning is for the limiting case of high 1 : 1 electrolyte to polyion concentration ratio as in the earlier "limiting laws" [3,4], and for a hypothetical line charge polyion. Thus it is of considerable interest to see how the degree of binding of earth alkaline and transition metal ions to a variety of polyions corresponds to this theoretical treatment. However, the number of experimental studies allowing such a comparison is very limited.

It is possible to study the binding process of the divalent ion indirectly by following the activity of the released univalent ions. However, it is far better to study the binding of the divalent ion directly. For this purpose, measurements in mixed counterion polyelectrolyte solutions with added salt have included Donnan exclusion measurements with a dialysis cell method [5,6], selective ion electrode methods [6], ESR [7], and dye binding spectrophotometry [8]. Selective ion electrode methods do not provide data which are accurate enough in the concentration range of physiological interest (typically well below 10^{-3} M for the divalent ion) and for some metal ion electrodes there can be considerable interference from the univalent ions present in large ex-

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cess. Donnan equilibrium measurements often suffer from relatively large errors caused by the analytical determinations, especially at low metal ion concentrations or when the amount of metal ion bound is small. ESR methods are of course applicable only to metal ions with unpaired electrons like the Mn^{2+} ion. The dye spectrophotometric method used in this study has the advantages of relative simplicity, applicability in the range of metal ion concentrations of physiological interest, and absence of interference by excess 1 : 1 electrolyte.

Dye spectrophotometric measurements for the determination of metal ion activities date back to the work of Raaflaub [9] and of Schwarzenbach [10]. Kohn and co-workers [11–14] have used a variation of this method, which we will call the two wavelength ratio method for an extensive study of earth alkaline metal ion activities in polysaccharide solutions. Krakauer [8] was the first to apply the method of Diehl and Lindstrom [15] which we will call the extrapolation method, to biopolymer solutions with mixed counterions and excess 1 : 1 electrolyte. It is the purpose of this paper to examine the applicability of the dye spectrophotometric method to the determination of divalent metal ion activities and free metal ion concentrations in polyelectrolyte solutions with added 1 : 1 electrolyte. A comparison of the methods of Kohn [11] and of Krakauer [8] will be made, and for well defined systems the results from the dye method will be compared with those obtained from the long established electrochemical or Donnan equilibrium methods, in concentration regions where both techniques provide reliable data. In a subsequent paper [16] the Kohn method will be applied to the study of Mg^{2+} and Ca^{2+} binding to dextransulfate in solution containing added NaCl or KCl.

In the dye spectrophotometric method a small amount of dye is added to the solution containing the divalent metal ion, polyion, and added electrolyte, the amount of dye being so small relative to the polyion monomolar concentration and the metal ion concentration that the fraction of metal ions taking part in the dye equilibrium

$$M + D \rightleftharpoons MD \qquad (1)$$

(M = metal ion, D = dye, MD = metal–dye complex) is small. Moreover, the formation constant of the metal–dye complex must have an appropriate value such that under actual experimental conditions the equilibrium in eq. (1) is neither fully to the right nor to the left. In practice this means that for activity ranges of physiological interest the apparent equilibrium constant K_{app}, defined by:

$$K_{app} = C_{MD}/C_M \cdot C_D \qquad (2)$$

should be between 500 and 10 000. The dye should have large extinction coefficients in both complexed and uncomplexed forms, and the extinction maxima of the two forms should be well separated. Finally, the dye should be relatively stable, and the absorption should be independent of solution pH, or the pH of the solution should be carefully controlled. In this study we use Eriochrome Black T (EBT) for the determination of magnesium activities [8] and tetramethylmurexide (TMM), a reagent used extensively by Kohn and co-workers [11–14], and in a different application by Ohnishi [17] for the determination of calcium activities. For TMM-Ca^{2+} the reported K_{app} values for an ionic strength range of 0.005–0.1 mole/ℓ are 500 [12] and 360 at pH 7 [17]. Since the TMM–Ca^{2+} equilibrium is pH independent K_{app} should not vary with pH at least in the pH range studied by these authors. For EBT–Mg^{2+} K_{app} values between 2000 [8] and 4000 [10,15] are reported in solutions of pH 8 and ionic strength 0.1 mol/ℓ. Since the molar absorbance of EBT solutions and the EBT–Mg^{2+} equilibrium strongly depend on pH, all solutions must be carefully buffered.

2. Method

Consider the dye–metal ion equilibrium:

$$M^{2+} + H_nD^{m-} \rightleftharpoons MD^{(m+n-2)-} + nH^+ \qquad (3)$$

for TMM $n = 0$ and $m = 1$; for EBT in the pH range 7.3 – 10.5, $n = 1$ and $m = 2$. The thermodynamic equilibrium constant, K_{Th} is given by:

$$K_{Th} = (C_{MD}/C_{H_nD})(\gamma_{MD}a^n_{H^+}/\gamma_{H_nD})a_M^{-1}, \qquad (4)$$

where C_{H_nD} and C_{MD} are the concentrations of free dye and of metal–dye complex, γ_{MD} and γ_{H_nD} their respective coefficients, a_{H^+} the hydrogen ion activity and a_M the free metal ion activity. For the sake of simplicity the charges on M, D and MD have been omitted. If we define a function K as:

$$K = (C_{MD}/C_{H_nD}) \cdot a_M^{-1} \qquad (5)$$

it is clear from comparing equations (4) and (5) that K will be a constant if $\gamma_{MD}/\gamma_{H_nD}$ and a_{H^+} are constant. Thus, K can be considered constant at a given ionic strength and a given pH, as long as the dye concentration is low relative to the ionic strength. a_M can be determined if C_{MD}/C_{H_nD} and K are known:

$$\log a_M = \log C_{MD}/C_{H_nD} - \log K. \tag{6}$$

At a given wavelength the total absorbance of HD and MD is given by:

$$A = \epsilon_{HD}C_{HD} + \epsilon_{MD}C_{MD} \tag{7}$$

where ϵ_{HD} and ϵ_{MD} are the extinction coefficients of HD and MD at the given wavelength. It can easily be shown [9,14] that:

$$C_{MD}/C_{HD} = (A^0 - A)/(A - A^E), \tag{8}$$

where A^0 is the absorbance in the absence of metal ion M, and A^E the absorbance in the presence of excess M. Once for a given solution A^0, A^E and A have been determined, a_M can be obtained from eq. (6) by using the appropriate value of K determined from A^0, A^E and A measurements of a reference solution of identical ionic strength and known a_M. Although this procedure appears simple, there are a number of problems. A long extrapolation is needed to obtain K from the absorbance measurements in the reference solutions, resulting in large uncertainties in K which in turn can seriously influence the activity determinations in the unknown. This problem is especially serious since K depends strongly on the ionic strength. The extrapolation method has been used by Krakauer [8] tc determine the free magnesium ion concentration in mixtures of $MgCl_2$, NaCl, and various polynucleotides, using EBT at pH 8.0 as the dye. The long extrapolation involved in this method can be avoided by following a different procedure for calculating a_M. From the measured absorbances for solutions of known ionic strength and a_M, the log C_{MD}/C_{HD} values can be calculated using eq. (8). A reference curve is constructed for log C_{MD}/C_{HD} versus log a_M (eq. (6)). From the measured absorbances of the unknown solutions log C_{MD}/C_{HD} is calculated and the corresponding a_M values are obtained from the reference curve. In this procedure it is important to use exactly the same ionic strength in reference and unknown solutions. We will call this procedure the modified extrapolation method. A second problem with the dye method is the decomposition of the dye. This is not a serious problem with TMM, but in the case of EBT in solution autooxidation causes a decomposition rate of about 2% per hour at room temperature which can lead to serious errors in the activity determination. Although this problem can partially be overcome by "sandwiching" absorbance measurements on reference and unknown solutions, it is completely eliminated by the two wavelength ratio method described below.

The two wavelength ratio method first suggested by Buddecke and Drzeniek [18] was later well investigated by Kohn et al. [11–14]. In this procedure the absorbance of the solution is measured at two wavelengths instead of at one as is done in the extrapolation method. The wavelengths chosen are normally the absorbance maximum of the free dye (λ') and the absorbance maximum of the metal–dye complex (λ''), although in some cases it may prove advantageous to choose wavelengths slightly away from the maximum. The ratio of absorbances A at λ'' and at λ', $\phi = A(\lambda'')/A(\lambda')$, is plotted against a_M for a series of reference solutions of known metal ion activity, with ionic strengths identical to the unknown solutions and divalent to univalent ion concentration ratios similar to the unknown solutions, but of course without polyelectrolyte. This plot can be referred to as the calibration curve. For unknown solutions a_M, the activity of the free divalent metal ion, can be obtained by again determining ϕ for these solutions, and then using the calibration curve to obtain a_M in the unknown:

$$\phi = (\epsilon''_{HD}C_{HD} + \epsilon''_{MD}C_{MD})/(\epsilon'_{HD}C_{HD} + \epsilon'_{MD}C_{MD}), \tag{9}$$

where ϵ'' is the extinction coefficient at λ'', the metal–dye complex absorption maximum, and ϵ' is the extinction coefficient at λ', the free dye absorption maximum. Combining eqs. (5) and (9):

$$\phi = (\epsilon''_{HD} + \epsilon''_{MD}Ka_M)/(\epsilon'_{HD} + \epsilon'_{MD}Ka_M) \tag{10}$$

and thus we see that ϕ is a function of the extinction coefficients of HD and MD at λ'' and λ', of the ionic strength, the pH (if $n \neq 0$, eq. (3)), temperature, and a_M. If ϕ is measured at fixed λ', λ'', I, T, and pH it is only a function of a_M for a given metal ion–dye combination. We first of all notice that if ϵ'_{MD} and ϵ''_{HD} are zero ϕ is linear in a_M. Of course this is hardly ever the case. In the absence of metal ion we have $\phi = \epsilon''_{HD}/\epsilon'_{HD}$, i.e. a value smaller than 1 and in the case of a good separatoin of absorbance maxima $\phi(a_M = 0)$ will be close to zero. When we have a large excess of metal ion ϕ approaches a constant value $\epsilon''_{MD}/\epsilon'_{MD}$, which will be much larger than 1. For low and intermediate a_M values ϕ will be approximately linear at a given ionic strength and pH, in general when $\epsilon'_{MD}Ka_M \ll \epsilon'_{HD}$, ϕ will be linear in a_M. Thus we can predict the general shape of the calibration curve. First of all in the C_M range from zero to excess M^{2+} ϕ will vary from $\epsilon''_{HD}/\epsilon'_{HD}$ to $\epsilon''_{MD}/\epsilon'_{MD}$. ϕ will initially the linear in a_M, but it will start to deviate from linearity as a_M increases and finally it will reach a constant maximum value equal to $\epsilon''_{MD}/\epsilon'_{MD}$.

Although the dye spectrophotometric method seems a sensitive and reliable method, there still are a number of uncertainties associated with it. Questionable are for instance the assumption that $\gamma_{MD}/\gamma_{H_nD}$ (eq. (4)) is the same in reference and unknown solution, the influence of small pH variations (typically of the order of 0.01 pH unit), the ionic strength dependence of the extinction coefficients, the possibility of polymer–dye interactions (of course for the case of polyanions we choose anionic dyes), the ionic strength calculation in the polymer solution where only the free metal ion is included in the ionic strength, and as always the thermodynamic impossibility of apparently measuring a single ion activity. With respect to this last point we note that the method indeed only seemingly determines a single ion activity. Inspection of eqs. (3) and (4) reveals that the small anion (i.e. Cl^- in our case) should be included, and that its presumably constant activity enters both numerator and denominator of eq. (4). Before applying the dye spectrophotometric method to a study of metal ion–polyion interactions (16) we will in this paper compare the spectrophotometric activity determinations to the thermodynamically well defined results from Donnan equilibrium and EMF methods.

3. Experimental

The polymer, sodium dextransulfate, average molecular weight 500 000, was obtained from Pharmacia, Uppsala, Sweden. Potassium dextransulfate was obtained from the sodium salt by dialysing against KCl [19]. The purification and concentration procedures have been described elsewhere [19]. Eriochrome Black T was obtained from G. Frederick Smith Co., Columbus, Ohi. It was further purified according to the method of Diehl and Lindstrom [15]. Purified tetramethyl murexide was kindly supplied by Dr. R. Kohn of the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Czechoslovakia. For maintaining a constant pH, reagent grade tris(hydroxymethyl) aminomethane neutralised with HCl to a pH of 8.00 was used as a buffer. The buffer concentration in all solutions was less than 2.5×10^{-3} M. There are two reasons for selecting this buffer. First, it has a good buffering capacity at pH 8 and the low concentrations (2×10^{-3} M) used, and second the corresponding cation of the base, tris(hydroxymethyl)-methyl ammonium is a fairly large ion and is not expected to interfere in activity measurements of alkali or earth alkali metal ions. All solutions were made in deionized water and all concentrations shown in various figures and tables are in mode/litre (M).

Absorbance measurements were performed with a Cary 14 UV/VIS recording spectrophotometer. The instrument was calibrated with each change of wavelength. 1 cm high precision optical cells were used.

All mixtures were prepared by weight from stock solutions of known concentration. The ionic strength of the reference solutions is simply calculated as the sum of the ionic strengths of the constituents ($MgCl_2$ or $CaCl_2$, NaCl or KCl, buffer). In the case of the unknowns, the procedure is more complicated and more uncertain, at least when the ionic strength is relatively low. The problem here is whether or not to take into account the bound counterions in the ionic strength calculation. Since our measurement in fact determines the number of bound counterions there is no straightforward answer to this. We adopt a procedure based on the observation of condensation theory [4,6] that for univalent counterions the net charge density parameter ξ_{net} equals 1, and for divalent ions it equals $\frac{1}{2}$. Depending on the divalent metal ion concentration solutions are divided into three regions with $\xi_{net} = 1$, $\frac{1}{2} < \xi_{net} < 1$, and $\xi_{net} = \frac{1}{2}$. As long as $\xi_{net} > \frac{1}{2}$ the free divalent metal ion concentration is considered zero, and univalent counterions are bound to make $\xi_{net} = 1$. When $\xi_{net} < 1$ all univalent ions are considered free. The ionic strength is calculated by adding the concentration of all free small ions and the buffer. Although this simple behaviour is different from what is actually observed, at low ionic strength it resembles the observed behaviour reasonably closely, and at high ionic strength there is a large excess of 1 : 1 electrolyte. Moreover, the results of the ratio method are not critically dependent on knowing the ionic strength.

The dye solution was added to both reference and unknown solutions just before carrying out the absorbance measurements. This is required in order to minimize problems due to decomposition especially of EBT. The dye concentration in all solutions was fixed at about 3×10^{-5} M.

For EBT–Mg^{2+} the absorbance was measured at 615 nm (λ') and 560 nm (λ''). For TMM–Ca^{2+} 530 nm (λ') and 490 nm (λ'') were used.

The Donnan exclusion measurements used for $MgCl_2$ activity determinations have been described in an earlier communication [6]. The internal solution contained $MgCl_2$, NaCl, and dextransulfate. The external solution contained $MgCl_2$ and NaCl. The internal and external solutions were equilibrated in closed glass containers with constant stirring for about 100 hours. After equilibration both internal and external solutions were analyzed for Mg^{2+} (complexometric titration) and for Cl^- (titration with $AgNO_3$). The concentration determinations were reproducible to ±0.1% for Mg^{2+} concentrations above 0.004 M and for Cl^- concentrations above 0.01 M, with progressively larger errors at lower concentrations. For this reason the Donnan exclusion measurements were performed in solutions with a polyion concentration between 0.002 and 0.01 moles/ℓ and the lowest divalent metal ion concentration used was 0.001 moles/ℓ. Donnan exclusion measurements yield the mean ionic activity of $MgCl_2$ in the unknown solution.

The EMF measurements used for the determination of Ca^{2+} activities were identical to the ones described in earlier communications [6,20]. Orion Ca^{2+} electrodes (Orion Research Inc, Cambridge, MA, U.S.A.) were used in combination with Ag/AgCl electrodes, yielding the mean activity of $CaCl_2$ in the unknown solution.

Single ion activities for Mg^{2+} and Ca^{2+} were calculated from the $MgCl_2$ or $CaCl_2$ mean activities determined by the Donnan or EMF methods, for direct comparison with the dye-spectrophotometric method using the MacInnes convention (see results section). Since the maximum ionic strength used was 0.05 M and the maximum Mg^{2+} concentration was 0.006 M, this is a reasonable procedure [20,22]. Systematic errors in this convention would not influence our conclusions since all comparisons are at similar ionic strengths, and all solutions have only small Mg^{2+} or Ca^{2+} equivalent fractions. All needed mean activity coefficients were interpolated from the data in Robinson and Stokes [23,20].

4. Results and discussion

Calcium and magnesium ion activities in the polyelectrolyte solutions were calculated from the dye-spectrophotometric data using the following procedures. In the extrapolation method [8,15] the K value (eqs. (6), (8)), is obtained by plotting $\log (A^0 - A)/(A - A^E)$ versus $\log a_M$ for a series of reference solutions of constant ionic strength. The metal ion activity coefficients in the reference solutions were calculated with the MacInnes convention:

$$\gamma_{M^{2+}}(I) = \gamma_\pm^3(MCl_2)/\gamma_\pm^3(KCl) \tag{11}$$

where $\gamma_\pm(MCl_2)$ and $\gamma_\pm(KCl)$ are the mean activity coefficients of MCl_2 and KCl in pure salt solutions at the same ionic strength I. At the low ionic strengths used in these studies the various single ion activity conventions would lead to the same results [24]. Moreover, in our measurements the major interest is in variations of the single ion activities, and these variations will always be relative to the same reference solution. The value obtained for K is then used with eqs. (6) and (8) to determine a_M in the polyelectrolyte solutions. The long extrapolation involved in this method to obtain K is a serious drawback. For instance, for EBT–Mg^{2+} at pH 8.00 (buffer concentration 2×10^{-3} M, dye concentration 3×10^{-5} M), in NaCl–$MgCl_2$ mixtures of a total ionic strength $I = 0.1$ M, we find $\log K = 3.82 \pm 0.10$. Under the same conditions Krakauer [7] finds $\log K = 3.67$, thus the two values are within experimental error. If we apply these two values e.g. to dextransulfate – NaCl–$MgCl_2$ mixtures of polyion concentration 0.01 M and $I = 0.1$ M the calculated magnesium activities differ by as much as 30% in the magnesium concentration range 5×10^{-4}M to 6×10^{-3} M, even though the variation in the magnesium activity with concentration as calculated with a particular K value is perfectly consistent and is practically identical with either of the two K values. In order to avoid this ambiguity, which is not due to the measurements but to the errors inherent in the calculation procedure, we use a modified extrapolation method. In this method rather than actually extrapolating the results for $(A^0 - A)/(A - A^E)$ to obtain K (eq. (6)), a reference curve of $(A^0 - A)/(A - A^E)$ versus a_M is plotted for the reference solutions, and a_M in the unknown solutions is obtained from the value of $(A^0 - A)/(A - A^E)$ in these solutions.

In the two wavelength ratio method the absorbances

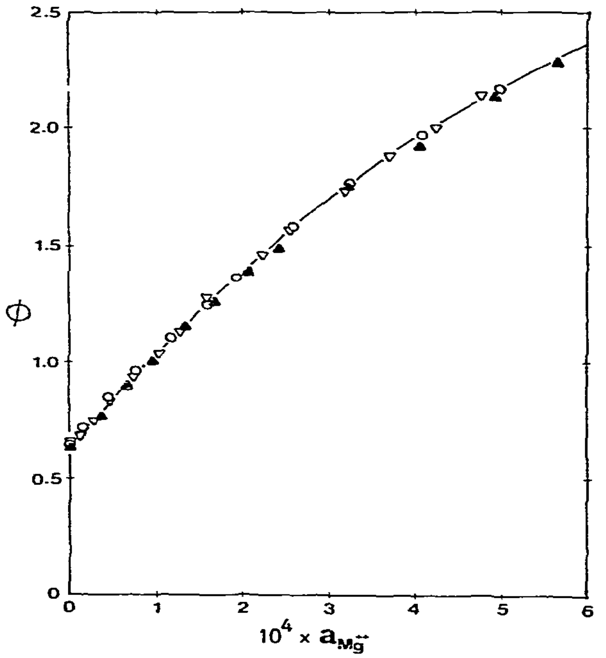

Fig. 1. The dependence of ϕ (eq. (10) on the Mg^{2+} activity in aqueous mixtures of NaCl and $MgCl_2$. Dye: EBT. pH: 8.00 ± 0.02. Total ionic strength: ▽: 0.01; ▲: 0.04; ○: 0.08. a_{Mg} calculated from eq. (11).

at λ' and λ'' of the reference solutions of fixed ionic strength and variable M^{2+} content are used to obtain a calibration curve of ϕ versus a_M. The a_M value in the unknown solution is then obtained from the ϕ value measured for this solution and the calibration curve at the same ionic strength. A typical example of calibration curves is presented in fig. 1 for a wide range of Mg^{2+} activities. In actual practice often only the lower range of the curve is needed, and many more points are used between 0 – 2 × 10^{-3} M than are plotted here. It is seen from fig. 1 that the ionic strength does not influence the ratio method calibration curve. The reproducibility of points on the curve is only moderate in this graph because the series at different ionic strengths were independently made up with different dye stock solutions. For actual measurements the same dye stock solution is used for reference and unknown solutions, and within the concentration range shown data points for ϕ and for a_M are reproducible to ±1% or better.

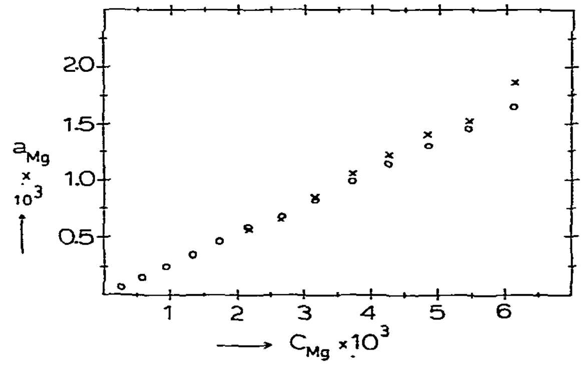

Fig. 2. Comparison of modified extrapolation method and two wavelength ratio method for Mg^{2+} activity determination in mixtures of dextransulfate, NaCl, and $MgCl_2$. Polyion concentration 0.001 M, ionic strength 0.015 M, pH 8.00 ± 0.02. ○: ratio method. ×: modified extrapolation method. Below $C = 2 \times 10^{-3}$ only ratio method points are shown, extrapolation method points are identical within accuracy of graph in this region.

In fig. 2 and table 1 we show comparisons of the results from the one wavelength extrapolated method

Table 1
Activity coefficients of Mg^{2+}, γ_{Mg}, in mixtures of dextransulfate, NaCl, and $MgCl_2$. $C_p = 0.001$ M, total ionic strength 0.015 M. Dye: EBT. pH: 8.00 ± 0.02. RM: ratio method; MEM: modified extrapolation method. Reproducibility of data points: ±1%.

	γ_{Mg}^{exp}	
$C_{Mg} \times 10^4$	RM	MEM
0.225	0.23	0.11
0.994	0.285	0.15
2.19	0.317	0.320
3.34	0.335	0.339
4.32	0.363	0.369
5.82	0.388	0.381
7.32	0.432	0.422
8.82	0.443	0.437
10.9	0.461	0.454
12.9	0.478	0.478
15.0	0.495	0.482
17.1	0.507	0.501

and the two wavelength ratio method. The results in solutions of high polyion concentration in fig. 2 were expressed as activities. The results for Mg^{2+} concentrations up to 4×10^{-3} M are within the estimated 1% precision, above this concentration the methods differ by 5 to 15%. We should point out that the a_M values for these two methods were calculated from two separate experimental runs, thus the errors include regular experimental errors in concentrations, absorbance, etc. On the whole the agreement between the two methods is very satisfactory especially in the range where we will do most of our experiments ($C_{Mg} < 4 \times 10^{-3}$ M). It is also clear from the graph that the scatter observed in the data points is far less for the two wavelength ratio method than for the one wavelength modified extrapolation method. In table 1 we compare results expressed as single ion activity coefficients of the Mg^{2+} ion, now for solutions of ionic strength 0.015 M, polyion concentration 0.001 M and Mg^{2+} concentrations between 2×10^{-5} and 1.7×10^{-3}M. Except for the first two points the agreement is within the estimated experimental error of ±1%. The first two points are at very low Mg^{2+} concentrations where the dye is still practically completely in the free dye form. In this region both methods lose sensitivity, just as they do in the very high Mg^{2+} concentration region where all dye is the metal-complex form. However, because the variation in ϕ is larger than the variation in $(A^0 - A)/(A - A^E)$ at one wavelength, the ratio method can be extended to lower and to higher concentrations than the extrapolation method. It is clear that the results from the ratio method are fully in agreement with those from the extrapolation method used by previous investigators, but the ratio method has a number of significant advantages. The ratio ϕ is independent of the dye concentration and therefore in the ratio method no significant error due to decomposition of the dye (e.g. the autooxidation of EBT) is introduced, and ϕ is not sensitive to small variations in dye concentration from sample to sample. With the extrapolation method autooxidation of EBT would result in calculated activities higher than the real values. The magnitude of this change would of course depend on the rate of decomposition and the measurement time. In the ratio method A^0 and A^E (absorbances in the absence of M^{2+} and with excess M^{2+} respectively) do not have to be determined. Finally, the ratio method is more sensitive than the extrapolation method, and can be extended to lower and higher metal ion concentrations. In our opinion the reproducibility and the accuracy of the activity determinations is also improved by using the two wavelength ratio method. In fig. 3 we compare activity coefficients of Mg^{2+} in mixtures of dextransulfate, $MgCl_2$, and NaCl as determined by the dye spectrophotometric method (ratio method) and the Donnan exclusion method. The comparison is done in a concentration region in which both methods can provide accurate data. It was shown earlier that for Mg^{2+} – EBT the ratio method can provide accurate data in the Mg^{2+} concentration range $2 \times 10^{-5} - 6 \times 10^{-3}$M. The Donnan exclusion method cannot provide accurate data if $C_M < 10^{-3}$M, largely because of analysis errors. In order to be able to do the comparison on solutions with Mg^{2+} to polyion concentration ratios in a range where the polyelectrolyte effect is particularly noticeable, we have chosen polyion concentrations C_p between 0.002 and 0.01 M, Mg^{2+} concentrations between 0.001 and 0.006 M, and ionic strengths between 0.004 and 0.05. The ionic strength cannot be closely controlled in Donnan equilibrium experiments. The solutions for the spectrophotometric

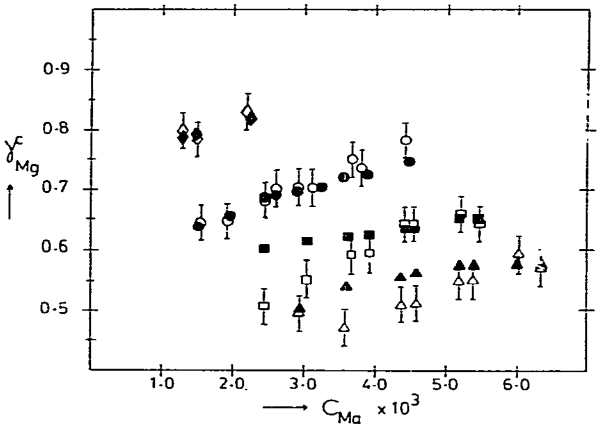

Fig. 3. Comparison of dye spectrophotometric method (ratio method) and Donnan equilibrium method for the determination of the corrected Mg^{2+} activity coefficient, γ^c in mixtures of dextransulfate, NaCl, and $MgCl_2$. ◇: $C_p = 0.002, I = 0.04$; ○: $C_p = 0.004, I = 0.04$; □: $C_p = 0.007, I = 0.05$; △: $C_p = 0.01$, $I = 0.05$. Open symbols: Donnan equilibrium method. Closed symbols: dye spectrophotometric method. Error bars indicate the estimated error in Donnan method. Estimated error in ratio method results is within the size of the data points as plotted.

method yields single ion activities. In order to compare the two methods for $MgCl_2$ mean activities calculated from the Donnan equilibration measurements were converted to Mg^{2+} single ion activities using the procedure of eq. (11). The Mg^{2+} activity coefficients presented in fig. 3, are corrected activity coefficients relative to the single ion activity coefficient of Mg^{2+} in the reference solution of the same ionic strength:

$$\gamma^{c}_{Mg} = \gamma^{exp}_{Mg}/\gamma^{0}_{Mg}(I), \qquad (12)$$

where γ^{exp}_{Mg} is the measured activity coefficient in the polyelectrolyte solution, i.e. $\gamma^{exp}_{Mg} = a_{Mg}/C_{Mg}$, and $\gamma^{0}_{Mg}(I)$ is the activity coefficient in the reference solution calculated with the MacInnes convention. In this case of excess salt this procedure will enable us to equate $(1 - \gamma^{c}_{Mg})$ to the fraction of Mg ions bound by the polyion [16]. When salt is not in excess, γ^{c}_{Mg} can be seen as representing the activity coefficient of Mg^{2+} in the polyelectrolyte solution corrected for the small ion–small ion interactions, i.e. the lowering of the activity due to polyion–small ion interactions only (i.e. binding + long range interactions). The measurement error in γ^{exp}_{Mg} as determined from the spectrophotometric measurements is estimated as ±0.006 in the concentraton range covered in fig. 3. This error estimate is based on the observed reproducibility of duplicate measurement series. The values calculated from Donnan equilibrium measurements are derived from single measurements, the error in $\gamma_{\pm}(MgCl_2)$ from the Donnan method, based on the uncertainties in the concentration determinations, is ±0.01. Of course this yields a much larger error in γ_{Mg}, estimated at ±0.03 for most of the values reported in fig. 3. The error bars in this figure are the estimated errors in the activity coefficients derived from Donnan exclusion measurements. The observed reproducibilities of the activity coefficients as measured with the ratio method are within the size of the data points in this graph. Fig. 3 clearly shows the superior reproducibility and consistency of the dye spectrophotometric data. We notice that within the estimated limits of error there is excellent agreement between the two techniques for the measurement series at $C_p = 0.002$ M ($I = 0.04$ M) and $C_p = 0.004$ ($I = 0.04$). Differences slightly outside the estimated error limits are observed for some of the points in the $C_p = 0.007$ ($I = 0.05$) and $C_p = 0.01$ ($I = 0.05$) series. For these two series the activity coefficients derived from the ratio method follow the expected concentration dependence, and the series are internally consistent. Thus the disagreement seems largely due to errors in the Donnan equilibration measurements. Generally the good correlation between the results from the two measurement methods reflects the reliability of the dye spectrophotometric data, corroborating the validity of the assumptions on which the method is based. In the systems under investigation EMF measurements with selective ion electrodes should yield metal ion activities with measurement errors comparable to or lower than the Donnan equilibrium measurements. We have found the Ca^{2+} liquid membrane electrode quite reliable in polyelectrolyte solutions with added NaCl [6]. We use the Ca^{2+} electrode in conjunction with a Ag/AgCl electrode, the electrodes are standardized on known NaCl–$CaCl_2$ mixtures as before [6]. γ^{exp}_{Ca} is again calculated from eq. (11). Dye spectrophotometric measurements for Ca^{2+} were done using TMM as dye and the two wavelength ratio method. Results for γ^{exp}_{Ca} from the two methods in dextransulfate-NaCl-$CaCl_2$ mixtures, $C_p = 0.001$, C_{Ca} varies from 4×10^{-4} to 1.2×10^{-3}, $I = 0.005$, are shown in fig. 4. For both measurement methods the estimated error in γ is indicated by size of the data points or by error bars. The complex formation constant K_{app} for the Ca^{2+}–TMM complex is almost a factor 10 smaller than the corresponding Mg^{2+}–EBT complex formation constant. Because of this the

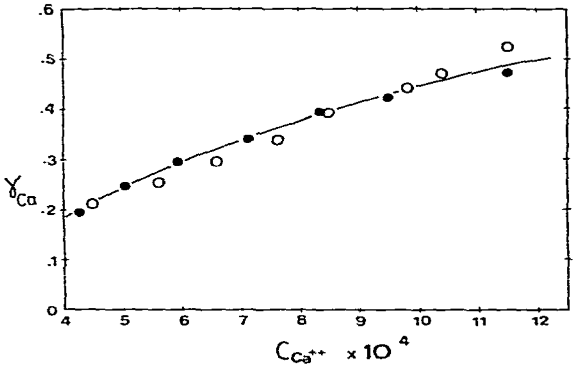

Fig. 4. Comparison of dye spectrophotometric method (ratio method) and ion selective electrode method for the determination of Ca^{2+} activity coefficients, γ^{exp}, in mixtures of dextransulfate, NaCl, and $CaCl_2$. $C_p = 10^{-3}$ M, total ionic strength 0.005 M. Dye: TMM. Open symbols: EMF method. Closed symbols: ratio method. Estimated error limits indicated by error bars or by size of the data points as plotted.

slope of ϕ with the Ca^{2+} activity is relatively small and as a result the reproducibility of the γ_{Ca} determinations with the dye spectrophotometric method is not as good as that of the corresponding γ_{Mg} determinations. We use an error estimate of ±0.01 for γ_{Ca} determined by the spectrophotometric method, and ±0.015 for data derived from EMF measurements. Finally, it should be noted that a Ca^{2+} concentration of 4×10^{-4}, corresponding to an activity of about 1×10^{-4} is certainly the lowest concentration where the Ca^{2+} selective ion electrode can still be used to obtain reasonably accurate values for γ. The ratio method on the other hand can be extended to Ca^{2+} concentrations well below 10^{-4} (i.e. activities as low as 10^{-5}), although here too the reproducibility is not as high as for the corresponding Mg activity determination. We observe a reasonable agreement between the two sets of data shown in fig. 4, with again the spectrophotometric data showing less scatter. Kohn et al. [11–14] have examined Ca^{2+} and Sr^{2+} activity determinations by the ratio method using TMM as dye, and also come to the conclusion that the ratio method can be used for accurate Ca^{2+} and Sr^{2+} activity determinations, sometimes even in the presence of Mg^{2+}.

In conclusion, we can state that the dye spectrophotometric method for Mg^{2+} and Ca^{2+} activity determinations yields accurate and reliable results even in complex mixtures containing polyions and excess univalent salts. The method is very suitable for the determination of activities in the range of physiological interest (10^{-5} – 10^{-3} M). Results from the dye spectrophotometric technique are in good agreement with data from classical techniques like Donnan equilibration and EMF methods. The dye spectrophotometric method yields data of superior reproducibility when compared with these two classical techniques, and can be applied to a wider range of solution concentrations for the metal ion of interest. From the two dye spectrophotometric measurement methods studied, the two wavelength ratio method developed by Kohn is found to be extremely convenient. Its results are not influenced by dye decomposition or by systematic errors due to long extrapolations needed to obtain the complex formation constant. The ratio method has an enhanced sensitivity when compared to the extrapolation method or the modified extrapolation method. For the ratio method or the extrapolation method to yield accurate data in mixed salt-polyelectrolyte systems a careful selection of the dye is needed. For instance, the K_{app} value, eq. (2), of the metal–dye complex should range approximately between 5×10^2 and 10^4. The dye should not interfere with the binding of counterions to the polyion. The absorbance maxima for the free dye and the metal–dye complex should be well separated, and the extinction coefficients of dye in the two forms should be large. These conditions are generally met for Mg^{2+}–EBT at pH 8, and for Ca^{2+}–TMM at pH 6–8.

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