For the polydispersity index I it may hold, then:

$$I = \frac{\Sigma J_i^{\frac{1}{a+1}} \cdot h_i \Sigma J_i^{-\frac{1}{a+1}} \cdot h_i}{(\Sigma h_i)^2} \quad (5)$$

equation 5 shows that the accuracy of I may be affected by the accuracy of the exponent a or by its variation with molecular weight.

To verify the effect of a upon I, we chose a model system, the linear calibration plot of which was determined by two points, namely, $M_1 = 10^6$, $V_1 = 90$ counts and $M_2 = 10^3$, $V_2 = 180$ counts. The universal calibration plot was also determined by means of a hypothetical Mark–Houwink equation, $[\eta] = 1 \times 10^{-4} M^{0.75}$. Hence the equation corresponding to the calibration straight line is:

$$\log J = 11.75 - 583\overline{3} \times 10^{-2} V_e \quad (6)$$

We also generated artificial chromatograms in the form of the Gaussian function (conclusions given below hold of course for any form of the chromatogram):

$$F(V) = \frac{1}{\sigma(2\pi)^{1/2}} \cdot exp\left(-\frac{1}{2}\frac{(V - V_{max})^2}{\sigma^2}\right) \quad (7)$$

where V_{max} is the elution volume of the maximum of the chromatogram. The standard deviation σ was varied so as to determine also the effect of width distribution, or in other words of the absolute value of I. Results of the calculations are summarized in *Table 1*. They show that for values up to $I \approx 1.1$ the choice of the exponent a is virtually without importance, because the change in I with a varying from 0.5 to unity lies within the limits of usual accuracy of g.p.c. (±5% relative). For the interval $1.4 < I < 2.5$, one can see that the error in the determination of the exponent $a \pm 0.05$ changes the resulting I again approximately within the limits of experimental error of g.p.c. Only at extreme values $6 < I < 23$ the error in the determination of I with the error $a \pm 0.05$ distinctly exceeds the limits of experimental error. In the case of linear calibration the values of I in *Table 1* do not depend on molecular weight, which may be proved by calculation after substitution of equation 6 into equation 5.

The correction of the polydispersity of I may be formulated e.g. by[8]:

$$I = e^{2D_2^2/h} \cdot I' \quad (8)$$

where I is the corrected value, I' is the uncorrected value, h is the correction factor and D_2 is one of the constants of the calibration function

$$M = D_1 e^{D_2 V_e}$$

defined as an inversion function to the linear function given above. For reasons of principle, the uncorrected value of I' calculated from g.p.c. data must be higher than the correct one. However, owing to the available accuracy of determination of the correct I by employing absolute methods, it seems useful to introduce correction only if the magnitude of this correction expressed as $(I - I')/I$ exceeds the experimental error of determination of I by absolute methods.

Thus, for instance, in the optimal case the error committed by us in the determination of M_w[9] in the range $M_w = 2.10^4$–2.10^6 is ±5%. M_n determined by membrane osmometry in the range $M_n = 2.14^4 - 3.10^5$ may in extreme regions be subjected to an error of ±10%. The minimum error of M_n values lies in the centre of this range, amounting to ±5%. M_n values up to 20 000 can be measured by vapour phase osmometry. Because of the concentration gradient in a drop of solution[10], the error of determination in the majority of solvents is approximately ±5% even at $M_n \approx 5.10^3$, while at the upper limit it amounts up to ±10%. Hence, I obtained from measurements employing absolute methods can in the best case be measured with an accuracy of ±10%.

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Acridine orange as a fluorescent probe for the study of polyelectrolyte complexes

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INTRODUCTION

The acidic sites of several polyanions can be titrated spectrofluorimetrically with metachromatic dyes such as acridine orange[1]. A stoichiometric complex containing one dye cation to one acidic site is observed in most cases. The dye can readily be displaced from such a complex by a more strongly binding

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species such as cetyl pyridinium chloride[2] or the polyamines spermidine spermine and polylysine[3], observed by pulse radiolysis and u.v. spectroscopy respectively. In the latter case[3], a one to one pairing of lysine residues with polyanion sites (DNA phosphate) was observed. The present work describes a preliminary application of the sensitive spectrofluorimetric technique[1] to the study of polyanion–polycation interactions using acridine orange as a probe. The method has the advantage that the fluorescence emission of this dye[4] is easier to analyse than the corresponding absorption spectrum which consists of several overlapping bands[5].

EXPERIMENTAL

λ, κ and *i*-carrageenans and carboxymethylcellulose (CMC) were supplied as previously reported[1]. Other polyanions were agar (Japanese, ex BDH), DNA (Calf thymus, highly polymerized, also BDH), heparin and chondroitin–4–sulphate (Sigma, London) and isotactic polystyrene sulphonate SPS (Dow Chemical Co., Midland, Michigan).

Poly(L-lysine hydrobromide) PLL (BDH Ltd.) was obtained at degrees of polymerization of 35 and 730. Lysine hydrochloride monomer was obtained from Sigma (London). Poly(*p*-xylyl viologen dibromide)(PPXV) was donated by Dr G. Ashwell, University of Nottingham, England.

Acridine orange was prepared as described elsewhere[1]. Fluorescence intensities were recorded on a fully corrected spectrofluorimeter. As previously reported[1], fluorescence intensities observed at 540 nm (excited at ~400 nm) are proportional to the concentration of monomeric dye in solution. The fluorescence of the dye bound to polyanions becomes shifted to longer wavelengths (λ_{max} ~ 650 nm). Addition of polycations has been shown[3,6] to restore the colour and absorption characteristics of monomeric dye in such systems. In this work the accompanying changes in dye emission are reported. All dye concentrations were 1.0×10^{-5}M. Both polyanion and polycation concentrations were measured in terms of ionic sites.

Table 1 shows the 'site equivalent masses' for the polymers used, the equivalent being the weight of polymer containing one charged group. The ratio of polyanion sites added to dye cations is recorded as the *P/D* ratio. Similarly the ratio of cationic sites to anionic sites is denoted by the ratio PC/PA. The fraction of free dye in solution, γ, is proportional to the emission intensity at 540 nm using right-angle viewing. A silica cell was used, fitted with a long stem to serve as a mixing chamber. Concentrated stock solutions (10^{-2}M dye, 10^{-3}M polyelectrolytes) were transferred to the mixing cell using a microlitre syringe as required.

Table 1 Sites and equivalent masses per ionic site for various polyelectrolytes

Polymer	Site type	Equivalent mass per site	Reference for structure
κ-Carrageenan	$-OSO_3Na$	408	7
i-Carrageenan	$-OSO_3Na$	255	7
λ-Carrageenen	$-SOS_3Na$	222	7
Chrondroitin-4-sulphate	$-CO_2Na$ $-OSO_3Na$	251.5	8
Heparin	$-CO_2Na$ $-OSO_3Na$ $-NH.SO_3Na$	178	9[a]
DNA	$-PO_4Na$	348	10[b]
SPS	$-SO_3Na$	206	–
CMC (DS 0.65–0.85)	$-OCH_2CO_2Na$	222	7
Agar	$-OSO_3Na$	~6400	c
PLL	$-\overset{+}{N}H_3\ Br^-$	209	–
PPXV	$>\overset{+}{N}-\ Br^-$	210	12

[a] For the purpose of defining an equivalent mass, this representative structure has been used. A great diversity of heparin structure is becoming increasingly evident, for example see ref 11

[b] An average weight for the nitrogenous base moiety was determined from the distribution of bases given in ref 10 for calf thymus DNA

[c] The equivalent mass from agaer was determined directly from dye binding measurements

RESULTS

Under the experimental conditions, lysine hydrochloride did not displace dye from polyanions even when the lysine was present in a large excess (200:1 lysine: AO). In contrast, the addition of poly(L-lysine hydrobromide), PLL, to dyepolyanion complex resulted in the immediate reappearance of the 540 nm emission of the dye (*Figure 1*). That some 80% of the binding curve was linear indicated a fixed stoichiometry which could be determined from the intercept *A* of this linear section of the dye-release curve with the line corresponding to 100% free dye. Stoichoimetries measured in salt-free solution are shown in *Table 2* for the nine polyanions and each molecular weight fraction of PLL. The complete binding of PLL to the polyanions required an excess of the polycation. Included in *Table 2* are values of the polycation: polyanion ratio at which the dye emission actually reached 100% (B in *Figure 1*).

That the excess polyanion sites in a polyelectrolyte complex are able to bind acridine orange is indicated in *Figure 2*, which shows the dye binding curves for premixed 2:1, 1:1 and 1:2 polycation: polyanion mixtures.

No dye binding is evident when PC > PA, but all of the excess sites when PA > PC were able to take up dye quan-

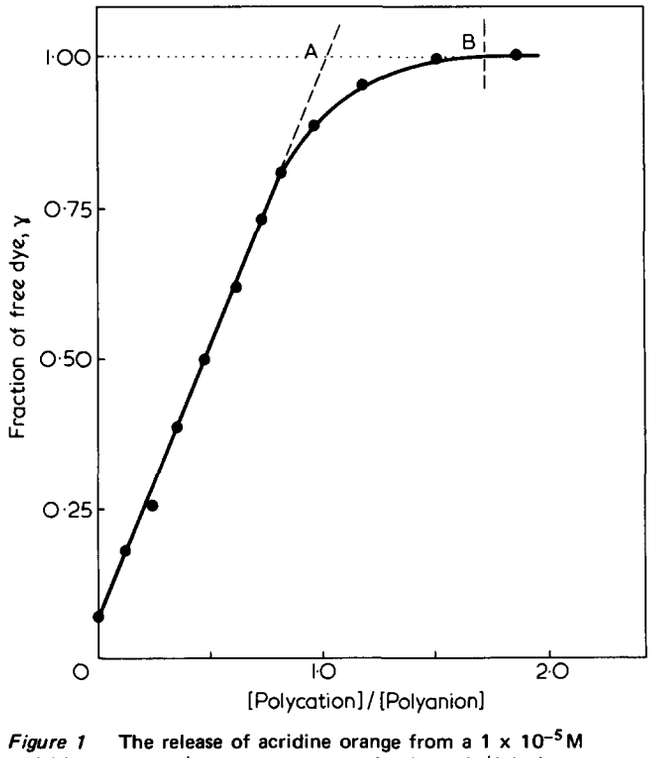

Figure 1 The release of acridine orange from a 1×10^{-5}M stoichiometric dye/κ-carrageenan complex by poly(*L*-lysine hydrobromide), (PLL)

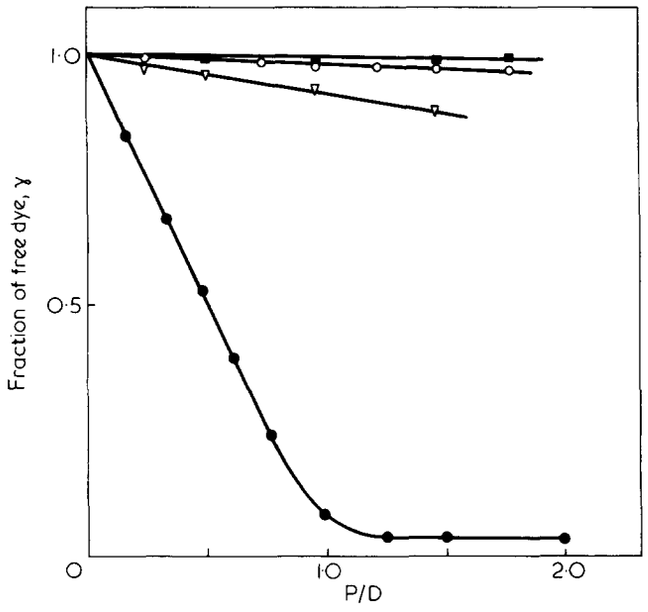

Figure 2 Uptake of acridine orange by premixed SPS – polycation complexes at SPS: PLL ratios 1:2 (○), 1:1 (△) and 2:1 (●) and the effect of polycation (■) on the 540 mm emission of acridine orange

titatively (*Figure 2*, closed circles). Polycation alone had no effect on the dye emission over this concentration range.

The nature of the dye cations which become fluorescent as PLL is added to dye-PA complexes (*Figure 1*) was investigated. The bimolecular quenching of acridine orange emission by sodium iodide can be used to discriminate between dye free in solution and dye bound to polyanions[13]. The former is strongly quenched by iodide ions and in the latter case repulsion between the iodide ion and the polyanion appears to reduce the quenching greatly. *Figure 3* shows results of quenching experiments in the form of Stern–Volmer plot. Iodide was added to the dye 'released' from 10^{-5}M dye-polyanion complexes by addition of a 2-fold excess of PLL. Results are expressed as Stern–Volmer quenching constants in *Table 3*.

DISCUSSION

The reappearance of the 540 nm emission peak on addition of polycations to dye–polyanion complexes enables the accurate determination of the stoichiometry of many polyelectrolyte complexes. The results presented here (*Table 2*) indicate that PLL forms complexes in which almost complete pairing of the lysine ϵ-amino groups and the acidic sites of the nine polyanions occurs, whether the acid site is a sulphate, carboxyl, carboxymethyl or phosphate group. The necessity for the displacing amine to be polymeric in order to give stoichiometric complexes is underlined by the failure of even a 2000-fold excess of monomeric lysine to produce appreciable dye-release. These findings are in agreement with those of Huse *et al.*[3], who observed that for a series of polyamines displacing the dye toluidine blue from DNA, the order of effectiveness was

PLL > SPERMINE > SPERMIDINE (>NaCl)

While the binding of each amine to polyanions has been considered as primarily electrostatic, it seems likely that the greatly increased binding affinity of polymeric amines over that of simple amines is related to the loss of translational energy, which would amount to some 3/2 kT (~4KJ/mole) for each immobilised molecule (k =Boltzmann constant). Assuming each amino group to be involved in binding at room temperature, these disruptive energy changes per mole of polyanion sites occupied would be approximately 114 joules for PLL of DP 35, whereas the simple amines indicated much larger values of 1000 joules (spermine), 1330 joules (spermidine) and 2000 joules (lysine). PLL of DP 730 behaved much the same as that of DP 35 (*Table 1*) indicating that above a certain DP value interaction becomes independent of molecular weight.

It would appear from the results of *Figure 3* and *Table 2* that the dye released by addition of polycations is essentially free in solution, i.e. little binding of the dye to either the polycation or polyelectrolyte complex is suggested, in contradiction to the findings of Huse *et al.*[3] who suggested that a possible PLL–dye interaction was occurring. Further, it is shown in *Figure 2* that excess polyanion sites are able to take up dye rapidly and stoichiometrically, as was also found for DNA/AO/PLL systems using pulse radiolysis[14]. The results with agar (*Table 1*) are interesting in that this polyanion has a very low degree of sulphation (5–10%). We have determined the degree of sulphation as ~5% by the dyebinding method[1], i.e. only one in twenty saccharide residues on average bear a charged group. Considerable flexing of the agar chains may occur to achieve complexes of the indicated stoichiometry. Such flexing would also be necessary to explain the properties of dye–polyanion complexes where dye–dye interactions appear to be essential to the binding process[4,15–18], and would require a small dye–dye separation. The spectral shifts in all dye–polyanion complexes are similar to those observed in concentrated dye

Table 2 Binding of PLL to polyanions at low ionic strength [AO] = [PA] = 1 x 10^{-5} M; no added salt

Polyanion	Stoichiometry of complex formation		Actual end points (*P/D* units)	
	1	2	1	2
λ-Carrageenan	1.2	1.2	1.6	1.7
κ-Carrageenan	1.2	1.2	1.6	1.7
i-Carrageenan	1.0	1.0	1.9	1.9
Heparin	1.0	1.1	1.0	1.15
Chondroitin-4-sulphate	1.0	1.0	1.1	1.1
DNA	1.1	1.1	1.2	1.2
SPS (Isotactic)	1.05	1.1	2.2	2.2
CMC	1.0	1.0	1.0	1.0
Agar	1.2	1.2	1.45	1.5

1 Poly(*L*-lysine hydrobromide) (DP = 35)
2 Poly(*L*-lysine hydrobromide) (DP = 730)

Table 3 Stern–Volmer quenching constants for 10^{-5}M acridine orange solutions containing stoichiometric polyelectrolyte complexes

Polyanion	Polycation	K_{SV} (M^{-1})
–	–	70
κ-Carrageenan	PLL	64
Chrondroitin sulphate	PLL	54
SPS	PLL	54
CMC	PPXV	67
Hyaluronic acid	PPXV	69

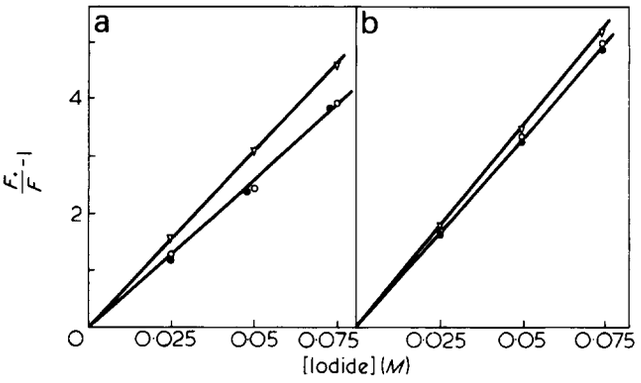

Figure 3 Stern–Volmer quenching curves of acridine orange released from dye–polyanion complexes by the polycations PLL(a) and PPXV(b). (a) ○, chondroitin sulphate; △, κ-carrageenan; ●, SPS. (b) ●, hyaluronic acid; ○, CMC; ▽, acridine orange

solutions, where a dye–dye separation as low as the Van der Waals distance (3.4 Å) has been proposed[19]. Thus, the binding of acridine orange may enable the polyanion to adopt a conformation suitable for subsequent binding to a polycation. However, recent evidence[20] suggests that agar is in fact a mixture of several fractions which can be separated on DEAE cellulose, and that certain of these fractions probably bear most of the ester sulphate groups. The agar fraction involved in electrostatic binding is therefore probably of higher degree of sulphation than the average value.

In conclusion, the present study has indicated that acridine orange is a sensitive probe for determining the stoichiometry of polyelectrolyte complexes. The cationic polypeptide PLL was observed to form complexes with polyanions in which the ratio PA:PC was always close to unity, irrespective of variation in structure, or type and number of the sites in the polyanion.

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