Interaction of Acridine Orange and Polyanions: Fluorimetric Determination of Binding Strengths and the Influence of Simple Electrolytes

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Binding affinities of Acridine Orange and six polyanions have been determined by measuring association constants in essentially salt-free solution, and also by observing competition between the dye and a simple salt for the polyanion site. Differences were found in the dye-binding order for the six polyanions using the two techniques. These differences were related to the breakdown in the uniform binding behaviour of carboxy and sulphate groups bound to the same polymer backbone as the salt concentration is increased. A mechanism is proposed which fits the thermodynamic parameters calculated by the application of Schwarz treatment and the Langmuir isotherm. The difference in the free-energy of binding between the strongest binding polymer, polystyrene sulphonate ($\triangle G^\circ = -41$ kJ mol^{-1}) and the weakest binding, hyaluronic acid ($\triangle G^\circ = -29$ kJ mol^{-1}) could be accounted for by considering coulombic site-dye interactions which destabilise the dye aggregates. The main influence promoting dye binding appears to be the free-energy of dye aggregation.

The degree of co-operativity of dye binding, q, has also been determined. An attempt has been made to relate this parameter to the physical properties of the polyanion chain. It appears unlikely that q has the significance initially attached to it.

THE interactions between cationic dyes and polyanions have long been of interest in cell staining where Scott [1] introduced the term ' critical electrolyte concentration ' (c.e.c.) as a measure of the strength of interaction between a particular polyanion and dye, the c.e.c. being that salt concentration required to prevent dye binding. Theory indicated [1] that the transition from the dye being bound to dye being unbound should be sharp, a prediction upheld by recent studies in these laboratories [2] although the values of c.e.c. varied with the technique employed.

The role of the metal cation in determining the c.e.c. value has been recently discussed,[3] and we report here c.e.c. values for 10^{-5}M-complexes of six polyanions with the cationic dye Acridine Orange, using fluorescence spectroscopy. It has been shown [4] that in polyanion-containing solutions, only two dye species exist at low polyanion to dye ratios. Free monomeric dye has an emission maximum at 525 nm, while binding of the dye to the polyanion is accompanied by a red-shift in emission with a new $\lambda_{max.}$ at *ca.* 640 nm and much reduced intensity. This gives a great advantage over the use of absorption spectroscopy where the dye spectrum consists of several overlapping bands which are difficult to resolve.[5] The fraction of free dye is proportional to the emission intensity at *ca.* 540 nm since there is no interference from bound dye, which does not emit at this wavelength.

A second aspect of dye binding which has received little attention due to the extreme sensitivity of the experimental techniques required, is the ability of the polyanion to maintain dye binding when the components are present in micro-molar concentrations or less. Jaques [6] has stressed that the ability of polyanions such as heparin to alter the activity of a small ion when present in trace amounts could well be crucial to the function of connective tissue glycosamino-glycans. The use of fluorescence spectroscopy affords such sensitivity of measurement, and we report the changing characteristics of the dye-binding profiles of six polyanions as the system is diluted.

Finally, the data has been analysed in view of current models for binding to a linear array of equivalent binding sites. The form of the Langmuir isotherm derived by Scatchard [7] and used by Peacocke and Blake [8] to describe the binding of acridine orange to DNA has been applied here to dye-polyanion systems. The model assumes n independent sites of intrinsic binding constant K_A'. If r is the amount of dye bound per mole of sites and $\bar{c}$ is the equilibrium free-dye concentration, then

$$r/\bar{c} = K_A' \cdot n - K_A' \cdot r$$

and a plot of $r/\bar{c}$ *versus* r should be linear, of slope K_A' and intercept n. A recent model due to Schwartz [9] assumes a linear array of equivalent sites with nearest-neighbour co-operativity, and describes binding in terms of (i) initial binding of an isolated dye (nucleation) with association constant $K^\ddagger$, followed by (ii) binding of a second dye adjacent to that bound monomerically, with association constant K_A. A third parameter, the degree of co-operativity, is defined as the ratio $K_A/K^\ddagger$.

The binding constants determined from such models are of special interest since they are more a function of the dye–polyanion system alone than the c.e.c. values, where some consideration of the relative affinity of each species for the polyanion ' site ' is necessary. The results presented here give some indication of those forces which contribute to the dye-binding process.

EXPERIMENTAL

Materials.—Carboxymethylcellulose 7 MP (CMC) was a medium viscosity grade of degree of substitution 0.7—0.8 (Hercules Powder Co. Inc.).

Sodium polystyrene sulphonate (PSS) was the isotactic conformer (Dow Chemical Co.).

Sodium heparin (Abbott Laboratories, Chicago) had an

anticoagulant activity of 150 USP units/mg. Other polyanions were Kappa carrageenan (Copenhagen Pectin Co.), hyaluronic acid (HA), and chondroitin sulphate (CS) [sodium salts from Sigma (London)].

Acridine Orange (AO) (dye-laser grade Kodak–Eastman was further recrystallised from ethanol. A 2.10^{-5}M-solution had ε 492 = 50 000 l mol^{-1} compared to a value of *ca.* 51 500 calculated by the equation of Bradley and Stone.[10] A stock solution of 10^{-2}M-dye was stable for up to one year when kept in the dark in a specially blackened flask. When required, dye was withdrawn from the stock solution with a microlitre syringe and subsequently diluted. The cells used in fluorescence measurements were fitted with a long neck and ground glass stopper so that adequate mixing of the cell contents could be accomplished. The inside cell surfaces were treated with Repelcote (2% dichlorodimethylsilane in carbon tetrachloride) (B.D.H.) to render the surfaces hydrophobic and reduce the amount of dye adsorption. This was a particular problem at very low dye concentrations.

Fluorescence Intensities.—These were recorded on a fully corrected instrument described elsewhere.[11] The use of this instrument in the study of AO–carrageenan binding has been previously reported.[4] The ratio of polymer sites added to dye cations in solution is expressed by the ratio P/D.

Critical Electrolyte Concentrations.—These were determined by pre-mixing P/D = 1.0 complexes of dye-polyanion, *i.e.* 10^{-5}M-dye containing 10^{-5}M-added polyanion sites. The free-dye fluorescence intensity was measured as a function of the added salt concentration. For low salt concentrations, the salt was added by microlitre syringe from a molar stock solution since volume changes were small and readily corrected. At higher ionic strengths, salt was added as the solid. Free-dye emission intensities were corrected for any dilution, and also for intensity changes due to salt-induced dye aggregation or quenching of the monomer emission by the chloride ion. The latter were effected using a calibration plot of dye emission at 540 nm *versus* ionic strength.

Dye Binding Profiles.—Such profiles as those shown in Figure 3 were obtained by addition of polyanion from a concentrated stock solution (10^{-3}M in sites), using a microlitre syringe, to a dilute dye solution in the cell. Profiles were plotted as fraction of free dye, γ, *versus* P/D.

The effect of progressively reducing the dye concentration, c^0, while varying the polyanion concentrations to give final ratio of P/D between 0 and 3 was investigated. Corrections were made for the amount of free dye which was aggregated in solution using a dimerisation constant [5] of $K_d = 1.1 \times 10^4$.

RESULTS

Salt Resistance of Complexes.—Figures 1 and 2 show the fraction of free dye γ, *versus* ionic concentration of NaCl and $CaCl_2$ for stoicheiometric 10^{-5}M dye–polyanion complexes. The sigmoidal shape of the curves is similar to that found for Methylene Blue–heparin complexes.[2] Almost quantitative dye-release occurs over two decades of logarithmic salt concentration for each of the six polyanions, and eventually reaches 100% in each case. C.e.c. values were calculated by extrapolation of the linear sections of the dye-release curves to 100% free dye. Values obtained are shown in Table 1 and indicate an order of binding strength:

$$\underbrace{\text{HA} < \text{CMC}}_{\text{carboxy}} \quad \underbrace{< \text{CS} < \text{Heparin}}_{\text{carboxy + sulphate}} \quad \underbrace{< \kappa\text{-Carrageenan} < \text{PSS}}_{\text{sulphate}}$$

Binding Strengths assessed by Dilution.—Dye-binding profiles were recorded over the range of P/D 0—3 at initial dye concentrations (c^0) between 3×10^{-7} and 5×10^{-5}M. Curves for one of the polymers, CMC, are shown in Figure 3. The curves for all six polyanions were qualitatively similar.

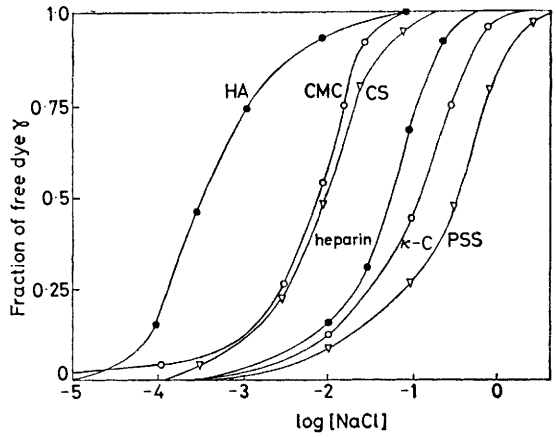

FIGURE 1 Variation of degree of dye binding with increasing sodium chloride concentration for 10^{-5}M, P/D = 1.0, Acridine Orange–polyanion complexes

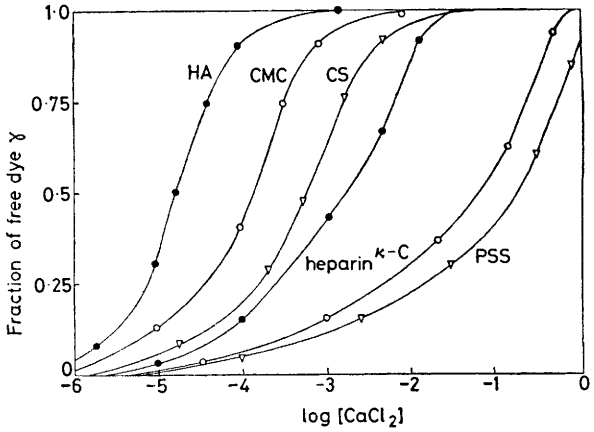

FIGURE 2 Variation of degree of dye binding with increasing calcium chloride concentration for 10^{-5}M, P/D = 1.0, Acridine Orange–polyanion complexes

At high c^0 a limiting behaviour was observed and the curve reduced to two linear sections, from which the number of binding sites per anionic group (g) could be determined from the intercept with the P/D axis. As c^0 was reduced, the

TABLE 1

Critical electrolyte concentrations for polyanion–Acridine Orange complexes from fluorescent intensity data

Polyanion	Critical electrolyte concentration (M) NaCl	$CaCl_2$
Hyaluronic acid	3.9×10^{-3}	8.5×10^{-5}
Carboxymethyl cellulose	3.7×10^{-2}	1×10^{-3}
Chondroitin sulphate	7×10^{-2}	6×10^{-3}
Heparin	0.26	2.2×10^{-2}
κ-Carrageenan	1.2	0.53
Polystyrene sulphonate	1.6	1.0

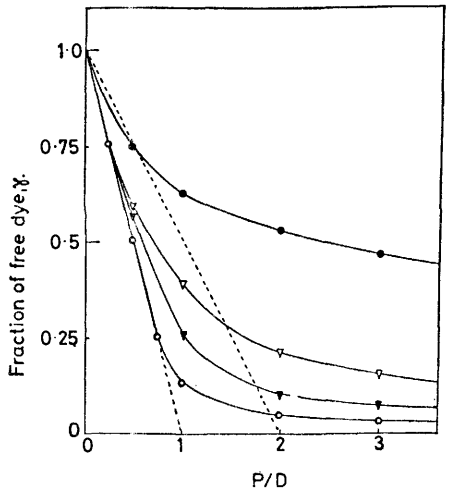

FIGURE 3 Variation of carboxymethylcellulose–Acridine Orange binding profiles between the range of P/D 0—3, at four initial dye concentrations: 1.0×10^{-6}M (●), 3.0×10^{-6}M (▽), 1.0×10^{-5}M (▼), and 2.5×10^{-5}M (○)

fraction of free ligand γ at a particular P/D increased progressively. Figure 4 shows how the fraction of ligand bound ($1 - \gamma$) varied with c^0 for a P/D = 1.0 complex, and shows the release of dye from the complex with dilution, but to a differing degree for each polymer. The apparent order of binding affinity can be seen to be: HA < CMC < κ-carrageenan < CS < Heparin ≪ PSS.

Models with and without Co-operativity.—Binding curves for each polyanion were analysed by the method of Schwartz.[9] As shown in Figure 3, a line is constructed of slope equal to half that of the binding curve at limiting behaviour, *i.e.* high c^0. The association constant for the binding process is then determined from the relationship $K_A = (\gamma c^0)^{-1}$ at the point where this line intersects the binding curves. Practically, c^0 of 3×10^{-6}M gave the most reproducible results, the value of γ being measurably large while c^0 was not so small that errors due to dye adsorption

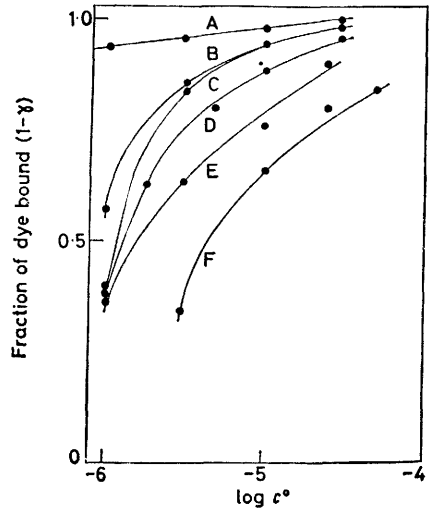

FIGURE 4 Variation of percentage dye binding in stoicheiometric polyanion–Acridine Orange mixtures with concentration of polyanion/dye in salt-free solution: A = polystyrene sulphonate, B = heparin, C = chondroitin sulphate, D = κ-carrageenan, E = carboxymethylcellulose, F = hyaluronic acid

on the cell walls became appreciable. Values of K_A and $\Delta G°$ (determined from $\Delta G° = RT \ln K_A$) are shown in Table 2.

TABLE 2

Binding constants, free-energy changes, and co-operativity parameters for polyanion–Acridine Orange complexes using the model of Schwartz [9]

Polyanion	c^0 $\times 10^6$	K_a $\times 10^{-6}$	$-\Delta G°$ / kJ mol^{-1}	q	g
Heparin	1	2	36.0	1.3—2.94	1.0
	3	3.2	37.1	3.33	
Hyaluronic acid	3	0.4	29.0	13—24	0.7
Chondroitin sulphate	1	1.67	35.5		
	3	3.3	37.2	7.15	1.0
κ-Carrageenan	3	2.08	36.0		
	10	2.29	36.3	9.62	1.0
Carboxymethyl cellulose	3	0.95	34.1		
	10	1.03	34.3	1.0—	0.7—
	1	1.3	34.9	1.7	0.8
Polystyrene sulphonate	1	16.4	41.16	1.0—18.7	
	0.3	18.5	41.46		1.0

For the polycarboxylates, sufficient free dye was present at P/D = 1.0 at most values of c^0 to allow evaluation of the Langmuir isotherm for these polymers directly. For

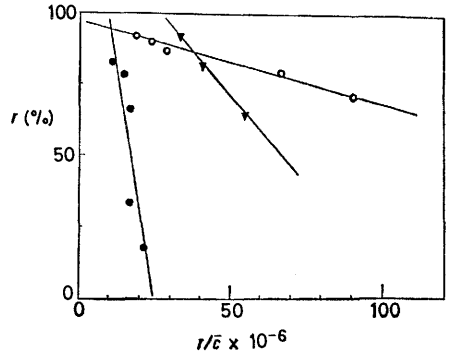

FIGURE 5 Langmuir-type isotherms for the binding of Acridine Orange to hyaluronic acid (●) carboxymethylcellulose (▼), and heparin (○)

heparin the isotherm was constructed by measuring the concentration of free dye $\bar{c}$ as a function of $c°$ for a P/D = 1.0 complex. The value of $\bar{c}$ was determined from the fluorescence intensity of the solution at 540 nm relative to a series of standard dye solutions. Figure 5 shows the resultant Langmuir isotherms for these three polyanions plotted as $r/\bar{c}$ *versus* r, where r is the percentage of dye bound at P/D = 1.0. Values of K_A' determined from the slopes of the isotherms in Figure 5 are shown in Table 3 together with values of $\Delta G°$ determined from $\Delta G° = RT \ln K_A'$. In each case the isotherm was reasonably linear over the whole concentration range studied.

TABLE 3

Association constants (K_a) and standard free-energy change ($\Delta G°$) of *P/D* I.O dye–polyanion complexes obtained from Langmuir isotherms

Polyanion	Dye	K_a	$-\Delta G°$ / kJ mol^{-1}
Hyaluronic acid	Acridine Orange	1.84×10^5	30.03
Carboxymethyl-cellulose	Acridine Orange	7.7×10^5	33.6
Heparin	Acridine Orange	3.7×10^6	37.5

Degree of Co-operativity.—The degree of co-operativity, q, was determined [9] from the slope of a plot of $\theta(1-\theta)/(1-2\theta)^2$ *versus* $s/(1-s)^2$ where θ is the fraction of occupied sites and is equal to $(1-\gamma)/(P/D)$, and $s = K_A \cdot \gamma \cdot c^0$ by definition. Co-operativity plots for two typical cases are shown in Figure 6. In general the slope of the curves either remained constant or increased with P/D. Values of q are shown in Table 2.

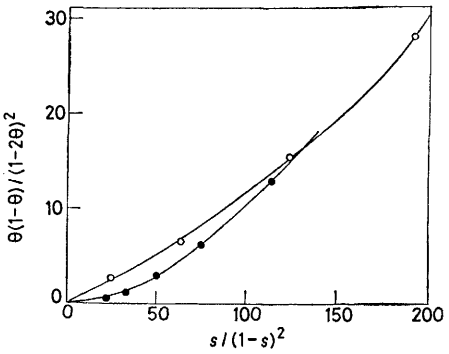

FIGURE 6 Representative co-operativity plots: chondroitin sulphate (○) and κ-carrageenan (●) at initial dye concentration (c^0) of 3.0×10^{-6}M

DISCUSSION

Dye–Polyanion Binding Affinities.—We have measured binding strengths of Acridine Orange and six polyanions using two fundamentally different approaches, firstly by measuring binding constants in essentially salt-free solutions and secondly by observing the competition between the dye and a simple salt for the binding site. The order of c.e.c. values given by the latter method (Table 1) is in good agreement with that found by other workers, indicating the generally higher salt resistance of sulphate-bound over carboxy-bound dye molecules.[1] The order of effectiveness of a series of ions in breaking dye–polyanion complexes [2] is $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} \gg K^+ > Na^+ > Li^+$ and has been proposed as being due to the stronger binding of the more effective ions to the site. Ions of higher valency are most strongly bound but within the same valancy group the most effective is that with the smallest hydrated radius. The stronger binding of a polarising metal cation such as Na^+ to a carboxy-group relative to a sulphate group has been suggested [12] to be due to an order of polarisability: $-CO_2^- > -PO_4^- > H_2O > -SO_4^-$. Hence, the solvated cation could accept a carboxy-group into its solvation shell, but not a sulphate group.

Binding affinity in the absence of salt determined by dilution methods indicate an apparent reversal in the ordering of κ-carrageenan (sulphate only) relative to heparin and CS (carboxy and sulphate). In both sodium and calcium chloride solutions, κ-carrageenan is the strongest binder, while in salt-free solution the order given by the free-energy changes shown in Table 2 is: heparin (1.75) ≈ CS (1.0) > κ-carrageenan (0.5) where figures in brackets refer to the charge density, *i.e.* the number of anionic groups per hexose unit. Good agreement can be seen between charge density and binding strength in the absence of salt, but this does not hold for the c.e.c. values. There are several possible explanations for this reversal. We have shown elsewhere [13] that in a salt-free solution the carboxy and sulphate groups of heparin appear to be identical as regards dye binding, in sharp contrast to the behaviour of a mixture of a polysulphate and a polycarboxylate where the sites behaved completely independently. Again, if the two types of site on heparin were to behave independently with respect to competing metal cations, we would anticipate a biphasic salt–dissociation curve for both heparins and CS complexes, a behaviour which was not observed (Figures 1 and 2). In view of the relatively low c.e.c. values for these two polyanions it is possible that the affinity of the metal ion for the carboxy-group weakens the dye binding, either by increasing the ionic strength within the domain of the polyanion, or by binding directly to the carboxy-site. The former explanation would seem more likely, since direct blocking of the carboxy-sites would still leave the degree of sulphation of heparin greater than that of the κ-carrageenan. It seems unlikely that direct binding of metal ions to carboxy-sites could interfere sterically with dye aggregated to the sulphate groups.

A further possibility is that the effect may be due to changes in the aggregation state of the dye in solution as the ionic strength is increased. The affinities, for example of the polyanions for monomeric and dimeric dye species may well be different, perhaps as a consequence of their varying intersite spacing which allow a better fit for these aggregated states.

It is difficult to make any definite conclusions as to the mechanisms of reversal in view of the vastly differing physical states of the systems at low and high ionic strength. Not only does the state of aggregation of the dye change as mentioned above, but the conformation of the polyanion will tend towards a less extended form due to shielding of the repulsive anionic sites by the salt. This will, in turn, affect the dye binding by altering the site spacing *etc.* The presence of salt also affects the structure of the solvent, which plays a crucial role in dye aggregation.[14] It is, therefore, difficult to compare the behaviour of the dye–polyanion systems in the presence and absence of added salt. One piece of evidence which may indicate a possible mechanism for the low c.e.c. values for hetero charged polyanions is the relative behaviour of heparin and CS. The two polymers are apparently similar in the absence of salt (Table 2), but from c.e.c. values (Table 1), heparin > CS. This seems to reinforce the idea that it is the attraction of the metal cation for the carboxy-group that destabilises the binding, since this would be predicted from the carboxy : sulphate ratios [15] of these polymers, 5 : 2 (heparin) and 1 : 1 (CS), that is the CS, with its higher fraction of carboxy-sites, is more affected by the addition of salt. However, it would be as easy to argue that, since the charge density of heparin is greater than that of CS, heparin may be a stronger binder of dye aggregates. The extent to which the contraction of the overall

dimensions of the polyanion affect site density is impossible to predict at this stage, but viscosity measurements on several polyanions have indicated that salt concentrations of *ca.* 0.1M cause large contractions.[16]

Mechanism of Dye Binding.—In a recent publication a mechanism for the binding of Methylene Blue to anionic celluloses was proposed.[3] The idea that the polyanion acts in some way as a template for dye aggregation by perhaps reducing coulombic repulsions between the cationic dye charges was furthered. The stronger binding of polysulphates relative to polycarboxylates was suggested to be due, in part, to the stronger attraction of the latter for the dye cation, which consequently reduced the possibility of dye–dye interactions.[3] The main driving force for dye binding was postulated to be the Gibbs free energy of dimerisation (−19 kJ mol^{-1}) of the dye, $\Delta G^\circ_{\text{sulphate}}$ being −22 kJ mol^{-1} and $\Delta G^\circ_{\text{carboxy}}$ being −20 kJ mol^{-1}. The data shown in Table 2 for the free-energy changes associated with AO binding is not as easy to explain in these terms. If we assume of free energy of dimerisation of −24 kJ mol^{-1} for AO, we can account almost totally for dye binding to HA (ΔG° = −29 kJ mol^{-1}) in terms of dimerisation, but ΔG° changes progressively for the other polyanions and reaching −41 kJ mol^{-1} for PSS. If the co-operativity, q, had increased as a function of K_A, it may have been possible to account for the increasingly negative ΔG° in terms of the formation of progressively longer dye aggregates, but as shown in Table 2 this was not found. The difference in ΔG° between the extreme cases of HA and PSS, was some 12 kJ mol^{-1}. We have attempted to account for such differences by considering coulombic site–dye interactions using the relationship given previously,[3,17] that is: where D is the dielectric constant of the solvent separating the ion and

$$-\Delta G_{el} = \frac{138.9}{D}\left[\frac{1}{r_e + r_d} - \frac{1}{r_e + r_m}\right]$$

site, and is again taken as 30 from the data of Rice and Nagasawa.[18] The effective spherical radius of the site, r_e, was calculated as 0.136 nm (carboxy) and 0.145 nm (sulphate) using the expression of Eisenman [19] and using a pK_A of 1.103 for carrageenan.[20]

The value of r_m, the sodium ion radius, has been proposed to resemble its crystal radius (0.095 nm) [21] at a carboxy-group and its hydrated radius at a sulphate group. Literature values of the latter vary considerably, and we have tried two values (0.216 [22] and 0.56 [17] nm) here.

The radius of the dye cation, r_d, is more difficult to assess, since it is not certain where the charge resides. Recent evidence suggests that it may be delocalised on the dimethylamino-group,[23] in which case the similar radius of the tetramethylammonium ion (0.347 nm) can be substituted.[22] A charge localised on the central ring nitrogen would have an effective radius similar to that of the ammonium ion (0.148 nm) [21] and this value of r_d has also been considered.

Calculated values for ΔG_{el} are shown in Table 4, and

TABLE 4

Calculated electrostatic free-energy changes for the ion-exchange of sodium and Acridine Orange cations at polyanion sites according to the relationship of Pauley [17]

Acid site	r_D/nm	r_M/nm	ΔG_{el}/ kJ mol^{-1}
SO_4^-	0.347	0.216	3.43
	0.347	0.56	−2.82
CO_2^-	0.347	0.095	10.46
SO_4^-	0.15	0.216	−2.82
	0.15	0.56	−9.07
CO_2^-	0.15	0.095	3.84

indicate that coulombic site–dye interactions could account on their own for the range of binding free-energies observed in Table 2, where a difference between HA and PSS of some 12 kJ mol^{-1} was observed. Whichever value of r_d is assumed above, a difference in ΔG_{el} between carboxy and sulphate group of up to 13—14 kJ mol^{-1} is evident.

It is, therefore, possible to postulate that the driving force for AO–polyanion binding is the large negative ΔG° of dye aggregation to dimer and higher aggregates, while differences in ΔG° appear to be associated with the contribution of coulombic site–dye interactions rather than the size of the aggregate.

The close agreement found between K_A values for CMC, HA, and heparin obtained from both Langmuir isotherms and Schwartz [9] analysis suggest that both theories describe the data adequately over the concentration range studied. The linear plots (Figure 5) from which we can assign an overall value of K_A' suggest that we are dealing with a uniform set of binding sites, while the applicability of Schwartz [9] method essentially indicates a set of sites with co-operative interactions. These two apparently conflicting pictures can be rationalised by considering the binding process to occur *via* either of the following mechanisms where aggregation is a necessary prerequisite to binding.

(1) An initial nucleation step could occur (comparable to the monomeric binding of dye in Schwartz treatment [9]) which is short lived unless stabilised immediately by the subsequent binding of an adjacent dye molecule. Binding of all dyes subsequent to the nucleation step would then occur with a similar free-energy change whether the site is a carboxy or sulphate, *i.e.* the ' binding site ' must be re-defined as an acidic group adjacent to an already bound dye cation. There has as yet been no conclusive evidence to suggest the existence of monomerically bound dye at low P/D ratios.

(2) A gradual increase in dye concentration is expected due to migration of AO cations in the polyanion electric field which would result in an increasing level of aggregated dye near the polyanion. Within the actual polyanion domain it would be unlikely that appreciable concentrations of monomeric dye exist, the AO being a very concentrated solution in this region. Binding to the polyanion could then occur *via* binding of dimers or higher aggregates to form longer bound aggregates. It is unlikely that the effect of simple electrolytes on the overall aggregation state of the dye in solution would

lead to the marked differences in the binding behaviour of κ-carrageenan, heparin, and CS observed in the previous section if this model were correct.

Degree of Co-operativity.—Values of q shown in Table 2 were very low compared to those obtained for other systems, although q tended to increase with P/D as found elsewhere.[24,25] The initial theory predicted that q would be independent of P/D, and this plus the indication in the preceding section and elsewhere that dye aggregation was the main energetic force for dye binding,[3] makes it unlikely that q has the physical significance originally assigned to it. In a recent approach [26] to co-operative binding, the formulae developed allow calculation of the average number of dye cations in an aggregate as $(1 + \sqrt{q})$. It will be seen that this fits well with our postulate of the initial nucleation step, as in the limit of non-co-operative binding (after Langmuir) $q = 1$ and there will be 2 dye cations on average in each aggregate.

It is apparent that the numerical value of q will be dependent on the strength of dye–dye interaction, and this will be influenced by such features as charge density and the intrinsic flexibility of the polymer chains. Bettelheim [27] has pointed out the difficulties of trying to evaluate the influence of these factors in particular cases, but some general points can be made. The five carbohydrates can be roughly divided into two groups. CMC and heparin both showed low q values, heparin being predominantly α-1,4 linked while CMC is all β-1,4. Carrageenan, CS, and HA had larger q values and are all alternating β-1,3/β-1,4 linked. Molecular models show that CMC has a large possibility for 0,5 to 0,3 hydrogen bonding giving a rigid ribbon-like structure which would have added stability since the carboxymethyl groups would be at maximum separation. Similar models for a member of the second group, κ-carrageenan, show that the 3,6 anhydride considerably reduces H-bonding possibilities and the chain is reasonably free to rotate about the glysocide linkages. Dielectric relaxation measurements [28] have shown that CMC chains undergo very little change in extension with dilution compared with κ-carrageenan suggesting a more folded, flexible conformation for the latter. For these two polymers, then, there are some grounds for suggesting that differences in the value of q can be related to the ability of the polymer to adopt a conformation whereby dye–dye interactions could be maximised.

Conclusions.—The results indicate that the initial step in dye binding must involve dye cations approaching the polyanion in such a way that each dye has at least one nearest neighbour dye species. The measured free-energy changes indicate that the affinity of the polyanions studied for AO is in the order PSS > heparin CS > κ-carrageenan > CMC > HA. The c.e.c. values showed a similar order but with the important change that now κ-carrageenan > heparin > CS.

This reversal can be accounted for in terms of the stronger attraction of carboxy-groups for metal cations, but may equally reflect that the two systems, *viz* dye/polymer/water and salt/dye/polymer/water are not amenable to direct comparison due to various activity and conformational changes at high ionic strength. Because of this there is no direct relationship between c.e.c. and binding affinity if the polyanions contain more than one type of charged group.

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REFERENCES

[1] J. E. Scott, *Biochem. Soc. Trans.*, 1973, **1**, 787.
[2] F. Jooyandeh, J. S. Moore, G. O. Phillips, and J. V. Davies, *J.C.S. Perkin II*, 1974, 1468.
[3] J. B. Lawton and G. O. Phillips, *J.C.S. Perkin II*, 1977, 38.
[4] R. B. Cundall, D. P. Rowlands, and G. O. Phillips, *Analyst*, 1973, **98**, 857.
[5] B. H. Robinson, A. Loffler, and G. Schwarz, *J.C.S. Faraday I*, 1973, **69**, 56.
[6] L. B. Jaques, in ' Polyelectrolytes and their Applications,' ed. E. Selingny and A. Rembaum, D. Reidel, Dordrecht, Holland, 1975.
[7] G. Schatchard, *Ann. New York Acad. Sci.*, 1949, **51**, 660.
[8] A. R. Peacocke and A. Blake, *Biopolymers*, 1968, **6**, 1225.
[9] G. Schwarz, *European J. Biochem.*, 1970, **12**, 442.
[10] D. F. Bradley and A. L. Stone, *J. Amer. Chem. Soc.*, 1961, **83**, 3627.
[11] R. B. Cundall and G. B. Evans, *J. Sci. Instr.*, 1968, Series 2, **1**, 305.
[12] H. J. Bungenberg de Jong, in ' Colloid Science,' ed. H. R. Kruyt, Elsevier, Amsterdam, 1949, vol. 2.
[13] R. B. Cundall, J. B. Lawton, D. Murray, and G. O. Phillips, *Die Makromol. Chem.*, 1979, **180**, 383.
[14] J. F. Padday, ' Chemistry and Molecular Biology of the Intercellular Matrix,' ed. E. A. Balazs, Academic Press, London, 1970, vol. 2, p. 1007.
[15] J. F. Kennedy, *Chem. Soc. Rev.*, 1973, **2**, 355.
[16] R. B. Cundall, J. B. Lawton, D. Murray, and G. O. Phillips, *Die Makromol. Chem.*, in the press.
[17] L. J. Pauley, *J. Amer. Chem. Soc.*, 1954, **76**, 1422.
[18] S. A. Rice and M. Nagasawa, ' Polyelectrolyte Solutions,' Academic Press, London and New York, 1961.
[19] G. Eisenman, *Biophys. J.*, 1962, 2 (Suppl.) 259.
[20] R. Sarkar, M.Sc. Thesis, University of Salford, 1974.
[21] L. Pauling, ' The Nature of the Chemical Bond,' Cornell University Press, 1967.
[22] R. H. Stokes and R. A. Robinson, ' Electrolyte Solutions,' Butterworths, London, 1968.
[23] S. K. Obendorf, J. P. Glusker, P. R. Hansen, and H. M. Berman, *Bio-inorg. Chem.*, 1976, **6**, 29.
[24] G. Schwarz and W. Balthasar, *European J. Biochem.*, 1970, **12**, 461.
[25] J. M. Menter, R. E. Hurst, and S. S. West, *Biopolymers*, 1977, **16**, 695.
[26] J. Applequist, *J. Chem. Educ.*, 1977, **54**, 417.
[27] F. A. Bettelheim, in ' Biological Polyelectrolytes,' ed. A. Veis, Dekker, New York, 1970.
[28] J. B. Lawton, unpublished results.