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ON THE BINDING OF ETHIDIUM BROMIDE IN SYNTHETIC POLYELECTROLYTE SOLUTIONS

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Abstract—Results are reported for spectroscopic and calorimetric studies of ethidium bromide (EBr) in water and in aqueous solutions of three alternating maleic acid copolymers. The dimerization constant, dimerization enthalpy and the dimer spectrum of EBr are derived. The interaction of EBr with the polycarboxylates is characterized on the basis of spectral and microcalorimetric data according to two limiting mechanisms: at low dye-to-polymer concentration ratios, EBr would be bound essentially in monomeric form with small exothermic effects: at high concentration ratios, the observed comparatively large exothermic heat is attributed to bound dye stacking.

INTRODUCTION

The interaction between ionic dyes and polyelectrolytes in dilute aqueous solution is a subject much investigated by various authors [1, 2]. Recently, the dye ethidium bromide has received considerable attention in view of the strong, selective interaction it can establish with native DNA under certain conditions [3].

No studies have been reported so far, however, on the physico-chemical properties of this dye in water and/or in aqueous solutions of synthetic polyelectrolytes. In this connection, one can mention only the brief statements of Lepecq and Paoletti [4] that "the absorption spectra of ethidium bromide in the presence of polyvinylsulphonate show that binding of the dye to this polyelectrolyte occurs with similar spectral changes as for DNA (but with no changes in the fluorescence characteristics)" and that "at high polyvinylsulphonate concentration, a decrease in the binding is observed (the spectrum reverts to that of the free dye), caused by the polyelectrolyte itself (ionic strength effect)".

In our opinion, a possible explanation of such findings lies in terms of a dimerization (or aggregation) of bound ethidium cations at low polyelectrolyte concentrations while, at relatively high polyelectrolyte concentrations, dye molecules would still be largely in the bound state but in monomeric form because of a "dilution" along the macroions chains.

This, of course, entails assumptions that: (a) the dimer spectrum of ethidium bromide is similar to the spectrum of the dye intercalated between adjacent bases of DNA (maximum absorption near 518 nm); (b) ethidium bromide, rather reluctant to dimerize in water in comparison to all other ionic dyes investigated so far, can dimerize when bound on a synthetic polyelectrolyte yielding a spectrum similar to that of the free dye-dimers; (c) the spectrum of the dye bound in monomeric form to an excess of polyelectrolyte is very similar to that of the free dye in dilute aqueous solution (maximum absorption at 480 nm).

We felt it worthwhile to investigate these points. To test assumption (a) above, and to characterize the behaviour of ethidium bromide in water, we have carried out a few spectral and heat of dilution measurements using dye solutions covering a range of concentrations as wide as allowed by the solubility of the dye in water (at 25°) and by the accuracy of the apparatus employed. Next, the interaction in dilute aqueous solution of ethidium bromide with three partially neutralized maleic acid (MA) copolymers has been investigated by means of spectral, equilibrium dialysis and microcalorimetric experiments.

Copolymers considered are: MA–ethylene (MAE), MA–propylene (MAP) and MA–isobutene (MAiB), which would also permit investigation of the possible influence of non-polar chain substituents on dye binding of polyelectrolytes having otherwise formally identical charge density and backbone chemical constitution.

The results reported here provide strong support for assumptions (a), (b) and (c) made above and afford original information on the enthalpy of dye binding in synthetic polyelectrolyte solutions.

EXPERIMENTAL

Maleic acid–propylene, maleic acid–ethylene and maleic acid–isobutylene copolymer samples were received from the Monsanto Chemical Company. The molecular weight of the samples, $\bar{M}_v$, was about 10^5. Aqueous solutions of the three maleic acid copolymers were prepared following essentially a procedure already outlined [5]. Solutions of the sodium salts of the copolymers were dialysed, passed through a cation exchange column in the H^+ form, dialysed again, concentrated under reduced pressure at *ca.* 40° and then titrated. The polymers were partially neutralized with tetramethylammonium hydroxide (BDH).

Tetramethylammonium perchlorate was prepared by direct mixing of concentrated solutions of $HClO_4$ (Erba, RP product) and of $(CH_3)_4NOH$. The white percipitate was washed with cold water and then with methanol, dried and then stored under vacuum. Ethidium bromide was a Sigma Chemical Company product, used without further purification.

The spectrophotometric measurements were carried out with an Hitachi Perkin-Elmer EPS-3t spectrophotometer. For low values of the ratio of the molar concentrations of polymer C_p (monomoles/l) to dye C_E^0, i.e. $C_p/C_E^0 < 5$, 2 ml of EBr solution ($C_E^0 = 1 \times 10^{-4}$) were put in a 1 cm path-length cell and a few μl of polyelectrolyte solution progressively added ($C_p = 10^{-2}$ monomoles/l). For higher C_p/C_E^0 values, the solutions were prepared separately in volumentric flasks.

The microcalorimetric experiments were carried out with a LKB 10700 batch microcalorimeter with glass cells [5]. It was established that, under the experimental conditions employed, no absorption of ethidium bromide on to the walls of the calorimeter cells took place.

RESULTS AND DISCUSSION

(I) *Characterization of ethidium bromide aqueous solutions*

The heat of dilution data for ethidium bromide (EBr) in water at 25° have been used to evaluate both the dimerization constant, K_D, and the enthalpy of dimerization, ΔH_D^0, of the dye. To this end, the necessary simplifying assumptions are that the heat of dilution of all EBr species is negligible with respect to the heat associated with dye dimerization and that no larger aggregates (i.e. trimers, etc.) are present. Consequently, the amount of heat Q given up by a volume V of an EBr solution (initial concentration $= C_E^0$, moles/l) with a concentration C_E^D of dimers and C_E^M of monomers given by:

$$C_E^D = (C_E^0 - C_E^M)/2 = K_D (C_E^M)^2 \qquad (1)$$

upon dilution in an excess of water (so that only monomers were present in the final state) is simply related to ΔH_D^0 thus:

$$\frac{Q}{V(C_E^0 - C_E^M)} = \Delta H_D^0/2 \qquad (2)$$

or:

$$\frac{Q}{VC_E^0} = \frac{\Delta H_D^0}{2} - \frac{QC_E^M}{VC_E^0(C_E^0 - C_E^M)}, \qquad (3)$$

which, on the basis of Eqn. (1), yields:

$$\frac{Q}{VC_0^E} = \frac{\Delta H_D^0}{2} - \frac{1}{2}\left(\frac{Q}{V}\right)^{1/2}\left(\frac{\Delta H_D^0}{K_D}\right)^{1/2}\frac{1}{C_E^0}. \qquad (4)$$

A plot of Q/VC_E^0 against $(Q/V)^{\frac{1}{2}}(1/C_E^0)$ for ethidium bromide is given in Fig. 1.

The linearity of such a plot lends support to the assumptions set forth above, and immediately yields, according to Eqn. (4), the following values of the thermodynamic parameters for the dimerization of EBr (in water at 25°): $K_D = 41$ (l mole^{-1}); $\Delta G_D^0 = 2{\cdot}19$ (kcal mole^{-1} of dimer); $\Delta H_D^0 = -7{\cdot}6$ (kcal mole^{-1} of dimer); $\Delta S_D^0 = -18$ (cal °K mole^{-1} of dimer). It is relevant that dimerization constants for other ionic dyes at around 25° in water or very dilute buffer solutions range from 5×10^2 (proflavine) [6] to 1×10^4 (acridine orange) [7].

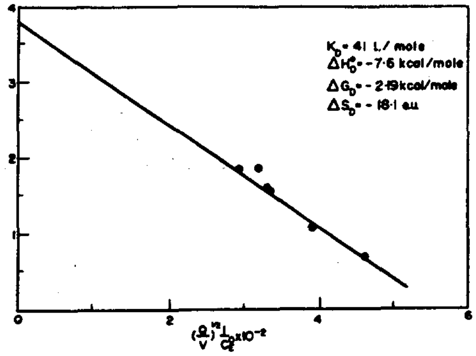

Fig. 1. Heat of dilution data for ethidium bromide in water at 25°. For the explanation of symbols see text [Eqns. (1–4)]. All the data are given per mole of dimer.

Dimerization enthalpies, however, seem to cluster around a mean value of $-6{\cdot}7$ kcal mole^{-1} (from van't Hoff plots of spectral data) from which individual figures for six different dyes, including ethidium bromide, do not differ by more than about 15 per cent [8].

Consider now the spectrum of ethidium bromide in water; Fig. 2 shows that the absorption maximum of the monomeric dye ($C_E^0 \sim 10^{-4}$) is centred at 480 nm with a molar extinction coefficient: $\epsilon_{480}^M = 5120$. To obtain the spectrum of dimeric EBr, a sample solution ($C_E^0 = 2{\cdot}33 \times 10^{-2}$) was put in a cell of path-length d (0·1 mm) and a reference solution ($C_E^0 = 1{\cdot}46 \times 10^{-4}$, concentration at which the dye is completely in the monomeric form) in a cell of path-length d' (1 cm) so that $C_E^M d = C_E^0 d (C_E^M$ in the sample solution was calculated knowing K_D) and the difference spectrum recorded. The results, also shown in Fig. 2 (dotted curve), lead to: $\lambda_{max} = 518$ nm and $\epsilon_{518}^D = 4290$ for dimeric EBr in water. It is interesting that the spectrum of EBr "intercalated" into the DNA double helix also exhibits a

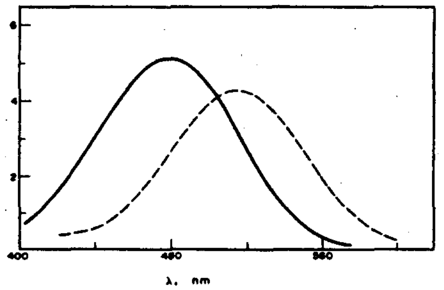

Fig. 2. Spectrum of ethidium bromide in water. Continuous curve; monomer spectrum. Dotted curve; dimer spectrum. (See text.)

maximum at 518 nm with a molar extinction coefficient of about 3800 [9]. It thus appears that, as far as the visible spectrum is concerned, similar perturbations are caused by partial overlap of the π electron system of either two EBr molecules or of one EBr molecule and the ring systems of two adjacent DNA bases. In both cases there is a net red-shift in the spectrum of the dye. Leaving aside the EBr–DNA case, we wish only to point out that such spectral feature is rather infrequent for dye self-association [10].

(II) *Interation of ethidium bromide with polycarboxylates*

(a) *Spectroscopic data.* Typical results of the spectral measurements carried out using aqueous solutions of EBr and half neutralized ($\alpha = 1{\cdot}0$) MAE and MAiB for different stoichiometric concentration ratio of polyelectrolyte to dye, C_p/C_E^0, are given in Fig. 3. The results obtained with the MAP copolymer ($\alpha = 1{\cdot}0$) are quite similar to those for MAE and are not reported.

Spectra of Fig. 3 show that, increasing C_p/C_E^0 up to near unity, the dye spectrum becomes hypochromic and is progressively red-shifted. For $C_p/C_E^0 \approx 1$, the spectrum resembles that reported in Fig. 1 for dimeric EBr in water. Under these conditions, at least a large fraction of bound EBr molecules should be stacked to form dimers (or higher aggregates) along the macroion chains. Data of Fig. 3 also show that the red-shift is larger with MAiB than with MAE, both at $\alpha = 1{\cdot}0$.

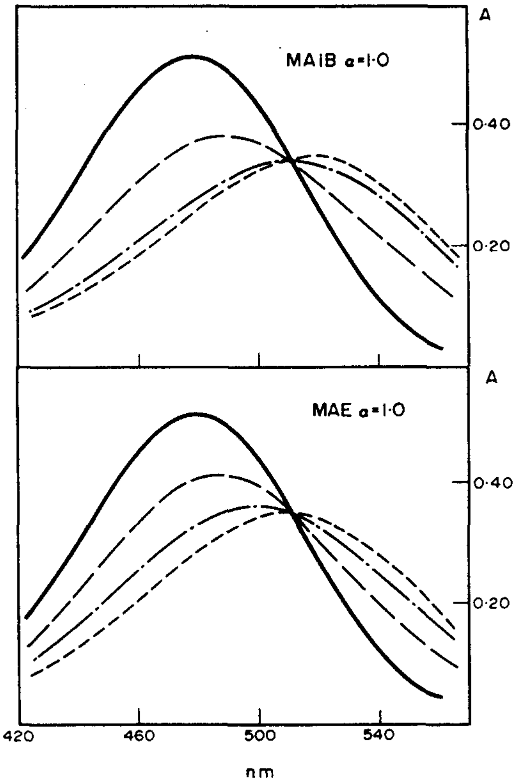

Fig. 3. Spectrum of ethidium bromide for various values of the ratio of polymer concentration (C_p, monomoles l^{-1}) to dye concentration (C_E^0, moles l^{-1}). MAiB and MAE were half neutralized ($\alpha = 1{\cdot}0$) with $(CH_3)_4NOH$. $C_E^0 = 1 \times 10^{-4}$ M. —— $C_p = 0$; --- $C_p = 0{\cdot}5 \times 10^{-4}$ monomoles l^{-1}; —·— $C_p = 1{\cdot}0 \times 10^{-4}$ monomoles l^{-1}; ----- $C_p = 1{\cdot}5 \times 10^{-4}$ monomoles l^{-1}.

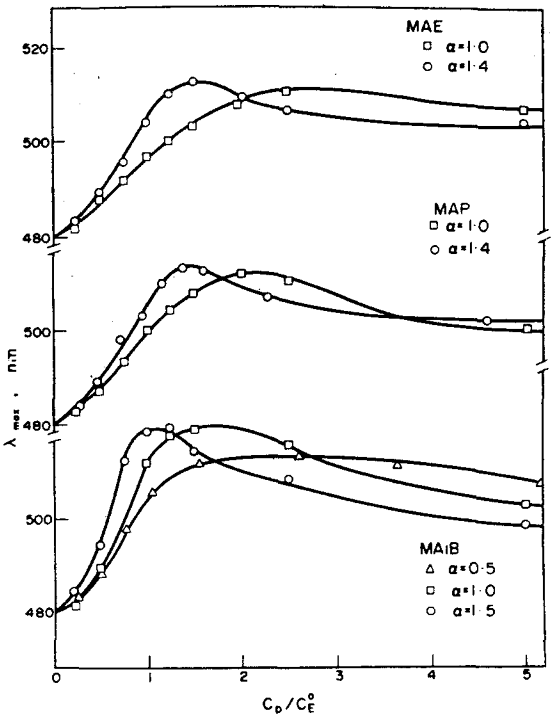

Fig. 4. Dependence of the wavelength of maximum absorption of ethidium bromide (λ_{max}) on the C_p/C_E^0 ratio (see Fig. 3). α is the degree of neutralization of the polyelectrolytes with $(CH_3)_4NOH$ ($\alpha = 2$ corresponds to full neutralization). In all experiments, the value of C_E^0 was kept close to 1×10^{-4} moles l^{-1}.

For $C_p/C_E^0 \gg 1$, the dye spectrum slowly reverts to that characteristic of the free monomeric form in water (see Fig. 1). This indicates that EBr molecules in the presence of an excess of polyelectrolyte would be bound essentially in monomeric form exhibiting a spectrum very similar to that of the free monomers. In all cases considered, the spectra show a clear isosbestic point at 510 nm, i.e. at the same wavelength as for the EBr–DNA system [9].

The results of the whole series of spectral measurements, are summarized in Fig. 4 in which the wavelength of maximum absorption of EBr λ_{max} (nm) is plotted against C_p/C_E^0 for the three copolymers considered at different α values. The plots of Fig. 4, besides clearly disclosing the different binding properties toward EBr exhibited by MAE, MAP and MAiB, show that the C_p/C_E^0 ratio at which the maximum red-shift occurs is a function of the degree of neutralization of the polyelectrolytes. More important, it is seen that if, as we assume, an increase of λ_{max} is indicative of a stacking of bound dye molecules, then such a phenomenon also takes place at low α values for which the spacings between fixed charges on the macroions is relatively large. In other words, dimerization of EBr on to the polyelectrolytes considered would probably occur through interaction of two dye molecules, only one of which is linked electrostatically to an ionized carboxylic group. The stacking tendency of EBr would be maximal when bound by MAiB ($C_p/C^0 \leqslant 1$) probably because the repeating units of this polymer would provide more hydrophobic sites for fixation of dye cations.

In this connection, the possible influence of a different sequence of configurations of the carbon atoms bearing the carboxylate groups along the chains passing from one copolymer to another should also be considered. Little is known, however, about this point, which merits further investigations.

(b) *Calorimetric data.* Binding of EBr to the polycarboxylates would occur both at low and at high values of C_p/C_E^0, but with different mechanisms, according to our spectroscopic data. To prove this independently and to gain a better insight of the energetics of EBr–polyions interactions, we have carried out a few microcalorimetric experiments.

To evaluate the enthalpy of EBr binding from data of heat of mixing dilute EBr solutions with half-neutralized (α = 1·0) polycarboxylate solutions, it was, of course, necessary to obtain additional information on the extent of dye–macroion association.

To this end, equilibrium dialysis measurements have been carried out. In these experiments (and consequently also in the microcalorimetric ones), $N(CH_3)_4ClO_4$ was present in all the solutions to reduce errors due to Donnan effects but at a concentration such that the same dye binding conditions as in the spectroscopic measurements would prevail.

It is found that only nearly equimolar concentrations of $(CH_3)_4NClO_4$ and of polyelectrolyte (α = 1·0) yielded essentially the same type of spectral results as those reported in Figs. 3 and 4. A higher salt concentration would have, in fact, very strongly reduced the extent of EBr binding, according to a few preliminary spectral and equilibrium dialysis experiments.

Consequently, our figures for the fraction of EBr molecules bound to the polycarboxylates are somewhat in error, due to residual Donnan effects. This should not invalidate a comparative analysis of the calorimetric data reported in Table 1. These data show that, for a nearly equal fraction of total EBr molecules bound by MAiB chains (C_E^B/C_E^0), the enthalpy of binding, ΔH_B, is only *ca.* −0·2 kcal/mole (mole of EBr bound) when $C_p/C_E^0 \sim 100$, but that ΔH_B is *ca.* −3·5 kcal mole^{-1} when $C_p/C_E^0 \sim 1$.

We consider these results as evidence in favour of a stacking of most EBr molecules bound by half-neutralized MAiB for C_p/C_E^0 near to unity. As a matter of fact, if we simply normalize the observed heats of interactions ($C_p/C_E^0 \sim 1$) per mole of pairs of bound EBr molecules we obviously get: ΔH_B *ca.* −7 kcal/mole of pairs; this value happens to be close to the enthalpy of dimerization of EBr in water (see Fig. 1).

Calorimetric measurements, carried out using MAE (α = 1·0) solutions and experimental conditions as specified in Table 1 for MAiB, have yielded the following results:

$$\Delta H_B = -2{\cdot}9 \text{ kcal mole}^{-1} \text{ for } C_p/C_E^0 \sim 1 \quad (C_E^B/C_E^0 = 0{\cdot}65)$$

and

$$\Delta H_B = -0{\cdot}8 \text{ kcal mole}^{-1} \text{ for } C_p/C_E^0 \sim 100 \; (C_E^B/C_E^0 = 0{\cdot}70).$$

Table 1. Calorimetric data on the interaction of ethidium bromide with half-neutralized maleic acid–isobutylene copolymer in aqueous solution (25°)

Soln. (1)*	Soln. (2)*	Heat of mixing (mcal)	Δq† (mcal)	C_E^B/C_E^0	ΔH_B‡ (kcal mole^{-1})
	Solvent: $(CH_3)_4NClO_4$ 1×10^{-2} M				
$C_p = 2 \times 10^{-2}$ (1·87)	Solvent (1·87)	0·612	—	—	—
$C_p = 2 \times 10^{-2}$ (1·84)	$C_E^0 = 2{\cdot}12 \times 10^{-4}$ (1·86)	0·652	0·050	0·70	−0·21
$C_p = 2 \times 10^{-2}$ (1·85)	$C_E^0 = 2{\cdot}12 \times 10^{-4}$	0·665	0·059	0·70	−0·21
	Solvent: $(CH_3)_4NClO_4$ 5×10^{-4} M				
$C_p = 2 \times 10^{-4}$ (1·89)	Solvent (1·90)	0·175	—	—	—
$C_p = 2 \times 10^{-4}$ (1·94)	$C_E^0 = 2{\cdot}02 \times 10^{-4}$ (1·97)	1·242	1·062	0·75	−3·6
$C_p = 2 \times 10^{-4}$ (1·86)	$C_E^0 = 2{\cdot}26 \times 10^{-4}$ (1·94)	1·284	1·112	0·73	−3·5
$C_p = 2 \times 10^{-4}$ (1·89)	$C_E^0 = 3{\cdot}06 \times 10^{-4}$ (1·91)	1·426	1·251	0·67	−3·3

* The number of cm^3 of solution (1) and (2) used in each mixing experiment is given in parenthesis.

† Δq is the difference between the heat of mixing dye with polyelectrolyte solutions and the heat of dilution of the polyelectrolyte (the heat of dilution of the dye was negligible).

‡ In kcal per mole of dye bound.

Consistent with the spectroscopic indications, the ΔH_B values suggest that binding of EBr by MAE leads to a smaller degree of dye-stacking than by MAiB, at C_p/C_E^0 near to unity, but to a higher degree of dye-stacking for higher C_p/C_E^0.

A logical extension of our investigation, and in which we are now engaged, is the systematic study of the fluorescence spectrum of EBr in dilute aqueous solutions of different synethetic polyelectrolytes, as this property seems to be particularly sensitive to the mode of binding for this dye [4].

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Résumé—On présente les résultats d'études spectroscopiques et calorimétriques du bromure d'éthydium dans l'eau et en solutions aqueuses de trois copolymères alternés de l'acide maléique. On a obtenu la constante de dimérisation, l'enthalpie de dimérisation et le spectre du dimère de EBr. On caractérise l'interaction entre EBr et les polycarboxylates en se basant sur des données spectrales et microcalorimétriques selon deux mécanismes restrictifs: pour des faibles rapports de concentration de EBr sur polymère, EBr se liera essentiellement sous la forme monomère avec de petits effects exothermiques; pour des rapports de concentrations élevés la chaleur exothermique relativement élevée qu'on observe pourrait être attribuée à une accumulation des liaisons de EBr.

Sommario—Vengono riportati i risultati di una serie di misure spettroscopiche e microcalorimetriche eseguite su soluzioni acquose du bromuro di etidio (EBr) in assenza e in presenza di tre diversi copolimeri dell'acido maleico (parzialmente neutralizzati). I dati sul colorante in acqua permettono la stima della costante di dimerizzazione, dell'entalpia di dimerizzazione e dello spettro delle specie dimere a 25°. La interazione del bromuro di etidio coi policarbossilati è quindi descritta in termini di due meccanismi limite: (I) associazione colorante–macroioni con gli ioni etidio quasi esculsivamente in forma monomerica e caratterizzata da una modesta esotermicità (alto rapporto tra concentrazione del polielettrolita e del colorante) e (II) aggregazione del colorante legato ai macroioni, caratterizzata da una comparativamente elevata esotermicità (basso rapporto tra concentrazione del polielettrolita e del colorante).

Zusammenfassung—Mitgeteilt werden die Ergebnisse spektroskopischer und kalorimetrischer Untersuchungen von Ethidiumbromid (EBr) in Wasser und in wässrigen Lösungen dreier verschiedener alternierender Copolymerer der Maleinsäure. Daraus wurden die Dimerisierungskonstante, die Dimerisierungsenthalpie und das Spektrum des dimeren EBr gewonnen. Über die spektroskopischen und die mikrokalorimetrischen Daten kann die Wechselwirkung von EBr mit den Polycarboxylaten auf zwei Mechanismen beschränkt werden: Bei kleinem Verhältnis von Farbstoff zu Polymerem wird EBr im wesentlichen in seiner monomeren Form in nur gering exothermer Reaktion gebunden; bei großen Konzentrationsverhältnissen wird die beobachtete vergleichsweise große exotherme Reaktionswärme einem stacking-Effekt zugeschrieben.