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Interaction between Ethidium Bromide and Various Polyelectrolytes and DNA with Emphasis on Spectral Characteristics and Binding Curves of the Bound Dye[1)]

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Visible and ultraviolet absorption spectra of ethidium bromide (EB) were measured by the optical titration method in the presence of each of three synthetic polyelectrolytes—sodium salts of poly(*p*-styrenesulfonate) (NaPSS), polyphosphate (NaPP), and polyacrylate (NaPA)—and native and heat-denatured DNA at 25 °C. By use of the principal component analysis (PCA) method, the number of EB species present in each EB–polymer system was determined to be two, *i.e.*, free and bound EB, in the range of the polymer residue-to-EB ratio between zero and about one. The spectrum and the fraction of EB species bound to each polymer were determined by the extended PCA method. The bound-EB spectra are bathochromic and hypochromic relative to the spectrum of free EB, but they show no metachromasy band. The apparent binding constants K_a for EB bound to polymers are in the order of NaPSS>denatured DNA>NaPP>NaPA≒native DNA.

In order to understand the general mechanism of metachromasy which results from the binding between dyes of particular classes and polyelectrolytes with highly charged sites, the pure spectrum of a given dye species bound to a particular polymer site, *i.e.*, the bound-dye spectrum, should be studied. This spectrum reflects the mode of interaction between the dye and the polymer site. Its extraction with a high resolution from observed absorption spectra of a dye–polymer system is, however, not an easy task because of the overlapping of the spectra of an unknown number of free and bound dye species and also because of the complicated reaction schemes with which the binding proceeds. It has been shown that the principal component analysis (PCA) method can be utilized to unravel the bound-dye spectrum.[2-8)] The bound-dye spectra of Crystal Violet (CV) and Trypaflavine (TF) show the characteristics of metachromasy, *i.e.*, the appearance of more than one absorption band, hypsochromism, and hypochromism.[3-6)] In the absence of polymers, CV and TF show a broad absorption band in the visible wavelength region, which consists of more than one electronic transition.[9,10)] A recent work on simpler acridine dyes, *N*-methylacridinium, and 9-aminoacridinium chlorides, has revealed that these bound-dye spectra exhibit only bathochromism and hypochromism.[5,7,8,11)]

Ethidium bromide (EB) has two amino groups at 3-, and 8-positions, but it belongs to a class of dyes different from 3,6-diaminoacridines such as Proflavine and TF. The interaction of EB with various biomolecules and biopolymers, particularly nucleic acids, has been studied by a number of physicochemical methods,[12-32)] but, precise information on the optical property of EB is still needed. In the present work, therefore, attempts were made to extract the bound-EB spectra by the PCA method. For this purpose, the spectroscopic interaction of EB with three polyelectrolytes with different functional groups and native and heat-denatured DNA was investigated in the low region of polymer residue-to-EB mixing ratio (P/D), where the binding scheme is rather simple. The empirical formula previously utilized for binding reactions was also improved upon to fit the thermodynamic concepts.

Experimental

Materials. Ethidium bromide (EB), 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, was purchased from Sigma Chemical Co. (St. Louis, Mo., USA). It was purified by the method of column chromatography. The three synthetic polyanions used in this work are sodium salts of poly(*p*-styrenesulfonate) (NaPSS, the degree of polymerization $DP=1360$),[3)] polyphosphate (NaPP, $DP=154$),[3,4)] and polyacrylate (NaPA, $DP=7700$).[3)] Calf thymus DNA was obtained from Worthington Biochemical Corp. (Freehold, N. J., USA).

Preparation and Measurement of Sample Solutions. Throughout optical titration measurements, the concentrations of EB were kept constant at about 7×10^{-5} and 1.5×10^{-5} mol dm^{-3} in the visible and ultraviolet regions, respectively. The concentrations were determined by using the molar absorption coefficient, ε, of 5850 mol^{-1} dm^3 cm^{-1} at 480 nm.[18,19,27)] No ionic additives, such as neutral salt and buffer reagent, were added to EB–polyanion solutions to avoid undesired side effects, while EB–DNA solutions contained 1×10^{-3} mol dm^{-3} NaCl to prevent denaturation. The hyperchromicity of DNA was about 34%.[33)] The concentration of native DNA (nDNA) solution was 9.35×10^{-4} mol dm^{-3}; this was determined by using the ε value of 6400 at 260 nm. Heat-denatured DNA (dDNA) was prepared by the heating-shock cooling method.[33)] Optical titrations were carried out by adding a stock polymer solution, prepared as before,[3,4)] to an EB solution in an absorption cell successively in about 20—60 μl portions with a graduated or a Gilson microburet. The mixture was stirred for a few minutes and its absorption spectrum was recorded at 25 °C.[3)] Volume corrections were made whenever necessary.[3,4)] The P/D ratio was defined as the concentration of polymer in monomer unit per concentration of EB present in a mixture.[33)] Path lengths of quartz cells were 2 cm in the visible and 1 cm in the ultraviolet region.

Data Analysis by the Principal Component Analysis. The principal component analysis (PCA) method has been described in detail[2-5)] and also in outline.[6)] By this method, the number of light-absorbing species, *i.e.*, *components*, can be determined. These components are responsible for serial changes in the absorption spectra of a given dye successively titrated with a given polymer in solution. When the number was two, the apparent equibrium constant, K, and the pure absorption spectrum, ε_λ^b, of the dye bound to the polymer

could be evaluated by the extended PCA method with the following equilibrium scheme:[3-8]

$$K = \frac{[DP^*]}{[D][P]^{\alpha}}, \quad (1)$$

where DP* is the dye–polymer complex or the bound dye in the low P/D region, D is the unbound or free dye, P is the polymer residue which is not occupied by the dye, the brackets indicate the equilibrium concentration of each species, and α is a single adjustable parameter. The fraction of the bound dye in a dye–polymer solution, f_b ($=[DP^*]/[D]_0$), may be calculated at any given P/D, $[D]_0$ being the initial concentration of the dye.[1-3,5]

Equation 1 is an empirical one which was introduced to explain observed optical titration curves for various dye–polymer combinations.[3-9] The problem associated with this formula is that the dimension of K is given by $[dm^3/mol]^{\alpha}$ rather than $[dm^3/mol]$. This irregularity is now resolved by dividing both side of Eq. 1 by $[D]_0^{1-\alpha}$, which is a constant, because optical titrations are carried out at the constant initial dye concentration. Equation 1 is rewritten as

$$K_a \equiv \frac{K}{[D]_0^{1-\alpha}} = \frac{[DP^*]}{[D][P]}\left(\frac{[P]}{[D]_0}\right)^{1-\alpha}. \quad (2)$$

According to Tanford,[34] the apparent association constant, k, for the ligand–polymer site may be expressed as

$$k = \frac{(DP^*)}{(D)(P)} = \frac{[DP^*]}{[D][P]}\left(\frac{\gamma_{DP}}{\gamma_D\gamma_P}\right)e^{-\phi(\tilde{\nu})} \quad (3)$$

where the parentheses stand for the activity, γ's for the activity coefficients, and $\phi(\tilde{\nu})$ is a general function for electrostatic interaction. It is interesting to note that Eq. 2 may be transformed into Eq. 3 by formally equating the appropriate factors:

$$\left(\frac{D}{P}\right)^{\alpha-1}\exp[(1-\alpha)\ln(1-\tilde{\nu})] = \left(\frac{\gamma_{DP}}{\gamma_D\gamma_P}\right)\exp[-\phi(\tilde{\nu})], \quad (4)$$

since $f_b = \tilde{\nu} \times (P/D)$ and since $[D]_0/P \equiv D/P$. Not entering into theoretical implications of this relationship in the present work, we shall discuss the experimental results by using values of K_a in Eq. 2.

Results and Discussion

Three EB–Polyanion Systems. A series of observed visible spectra of EB–NaPSS solutions are shown in Fig. 1 as an example. These spectra reveal two isosbestic points, at 513 and 395 nm, in the low P/D range. By dropwise addition of NaPSS, the peak of EB at 480 nm decreases and concurrently shifts toward the long wavelength. This spectral behavior is also observed for EB–NaPP (isosbestic points 527 and 395 nm) and EB–NaPA (516 and 392 nm) solutions. A similar result has been reported for the EB–NaPP system.[18] Optical titrations of EB are shown in Fig. 2, where values of ε_{480} of EB–polyanion solutions are plotted against P/D. Values of ε_{480} decrease less for NaPA and NaPP than for NaPSS. In any case, they do not decrease linearly but are curved, the curvature being convex at the initial P/D (<0.5). This sigmoidal trend in titration curves has already been pointed out.[3-5]

The presence of isosbestic points in each EB–polyanion system indicates that this may be a binary system, but the possibility that it contains three or more independent components cannot be excluded. (If the molar absorption coefficients of these components have an identical value at some wavelength, this would be an isosbestic point.) By applying the PCA method to each EB–polyanion system, the exact number of components was determined. The results are shown in Fig. 3, where ratios of the eigenvalue of the ith component to the largest eigenvalue of the *first component*, Λ_i/Λ_1, are plotted on a logarithmic scale. A large gap is seen between the second and third components in each case; the ratio of $\Lambda_1 : \Lambda_3$ is larger than $10^5 : 1$. These results confirm that each EB–polyanion system contains two absorbing species. Thus, it is reasonable to conclude that observed spectral changes are an interplay of two EB species, *i.e.*, free EB and EB–polyanion complex (bound EB),

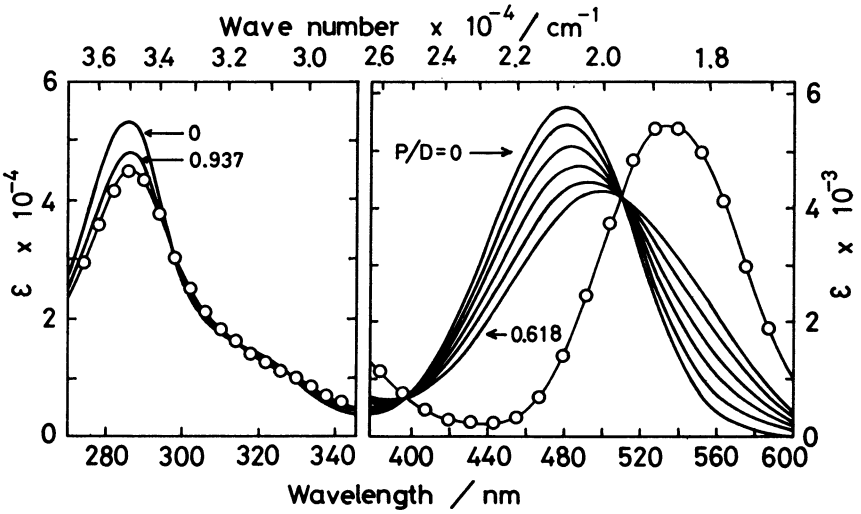

Fig. 1. Visible and ultraviolet spectra of EB titrated with salt-free NaPSS solution. The ordinates are the apparent molar absorption coefficients, ε, expressed in $mol^{-1}\,dm^3\,cm^{-1}$. Values of P/D are 0 (EB alone), 0.125, 0.243, 0.368, 0.493, and 0.618 in this order for the right, while they are 0 and 0.937 for the left. The concentration of EB$=7.47\times10^{-5}$ mol dm^{-3}. Open circles represent the molar absorption coefficients, ε_λ^b, or the bound-EB spectra.

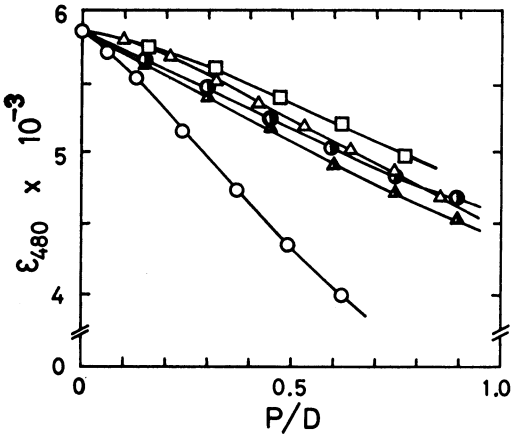

Fig. 2. Optical titration of EB with five different polymer solutions. The ordinate is the value of ε at 480 nm where the free EB shows the absorption peak. The abscissa is the mixing ratio. (○) NaPSS, (△) NaPP, (□) NaPA, (◑) native DNA, and (◭) heat-denatured DNA.

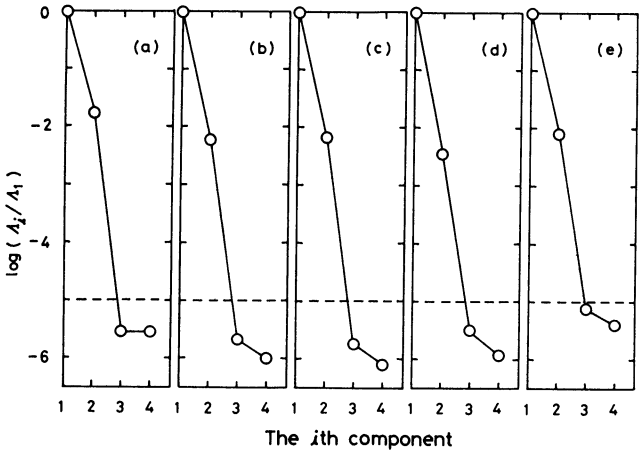

Fig. 3. Variations in the eigenvalue Λ_i with the ith light-absorbing component for five different EB–polymer systems. (a) NaPSS, (b) NaPP, (c) NaPA, (d) native DNA, and (e) heat-denatured DNA. The ordinate is the ratio of the eigenvalue of the ith component to that of the first. Dashed lines represent the upper limit to any significant components and are set under due considerations of experimental conditions.[2]

TABLE 1. THE APPARENT EQUILIBRIUM CONSTANTS, K_a AND K', AND THE EMPIRICAL PARAMETER α FOR EB–POLYMER SYSTEMS IN THE LOW P/D RANGE

Systems	α	$K_a \times 10^{-4}$ / dm³ mol⁻¹	$K' \times 10^{-4}$ a) / dm³ mol⁻¹
EB–NaPSS	1.4	9.9	6.6
EB–NaPP	1.7	1.4	1.0
EB–NaPA	1.4	1.2	1.0
EB–nDNA	1.1	1.2	1.1
EB–dDNA	1.1	1.6	1.5

a) $K' = K_a\left(\frac{[P]}{[D]_0}\right)^{\alpha-1}$. Values of K' were calculated at $P/D=1$.

in the low P/D range.

The Extended PCA Procedure and Bound-EB Spectra. On the basis of the above conclusion, Eq. 2 was used for evaluating the apparent equilibrium constant K_a and the empirical parameter α. The results are summarized in Table 1. Values of α are larger than unity for three EB–polyanion systems, corresponding to the convex curvature observed for titration curves in Fig. 2.[3-7] Values of the commonly-postulated apparent equilibrium constant, $K'=[DP^*]/[D]\cdot[P]=K_a([P]/[D]_0)^{\alpha-1}$, were computed at a convenient P/D value, *i.e.*, $P/D=1$.[3,4,6-8] They are given in Table 1, so that they may be compared with values reported for other dye–polymer systems.

With the optimized elements of the **t** matrix [Eq. 5 in Ref. 3], the spectrum of bound EB in the EB–NaPSS complex was calculated in the visible region (circles in Fig. 1). The spectral change of EB in the ultraviolet region is small, when a polyanion solution is added, and the strong absorption of free EB dominates the 340—235 nm region; hence, application of the extended PCA procedure is restricted. The ultraviolet spectrum of bound-EB, ε_λ^b, was therefore calculated with the fraction of bound EB, f_b, as

$$\varepsilon_\lambda^b = \varepsilon_\lambda^f + \frac{\varepsilon_\lambda - \varepsilon_\lambda^f}{f_b}, \tag{5}$$

where ε_λ^f and ε_λ are the molar absorption coefficients at λ of EB and EB–polymer solutions, respectively. The ultraviolet spectrum of EB bound to NaPSS is shown in Fig. 1. It is hypochromic relative to the spectrum of free EB.

The bound-EB spectra of three EB–polyanion complexes are summarily shown in Fig. 4, and their spectral characteristics are given in Table 2. These visible spectra are bathochromic and hypochromic to different degrees. The bathochromic shift is about 37 nm (NaPA)—55 nm (NaPSS). The hypochromism of bound-EB spectra was expressed with the absorption maxima, $1-(\varepsilon_{max}^b/\varepsilon_{max}^f)$, and also with the oscillator strengths, $1-(F^b/F^f)$. When EB is bound to a charged polymer site, neither band-broadening, new peak, nor shoulder seems to appear in the bound-EB spectrum above 400 nm; the number of transition moments probably remains unchanged, as judged both from values of the half-intensity band width ($\Delta\tilde{\nu}_{1/2}$) and from values of the oscillator strength F. Since the 480 nm band of free EB is due to a single electronic transition,[17,35,36] the same probably holds for the visible band of bound EB. It may be concluded from the data in Table 2 that EB bound to various polyanions exhibits neither the metachromatic blue shift nor the metachromasy band. This conclusion is based on the fact that the bound-EB spectra were obtained in the low P/D range, where possible dye-stacking would be maximum, and lead to the most pronounced metachromatic behavior, if any. Hence, possessing two amino groups, EB differs markedly from many 3,6-diaminoacridine dyes[3,4,18,37,38] but resembles the unsubstituted acridinium or 9-aminoacridinium chloride.[7]

Spectral Characteristics of EB–DNA Complexes. In order to obtain the spectrum of EB bound to DNA,

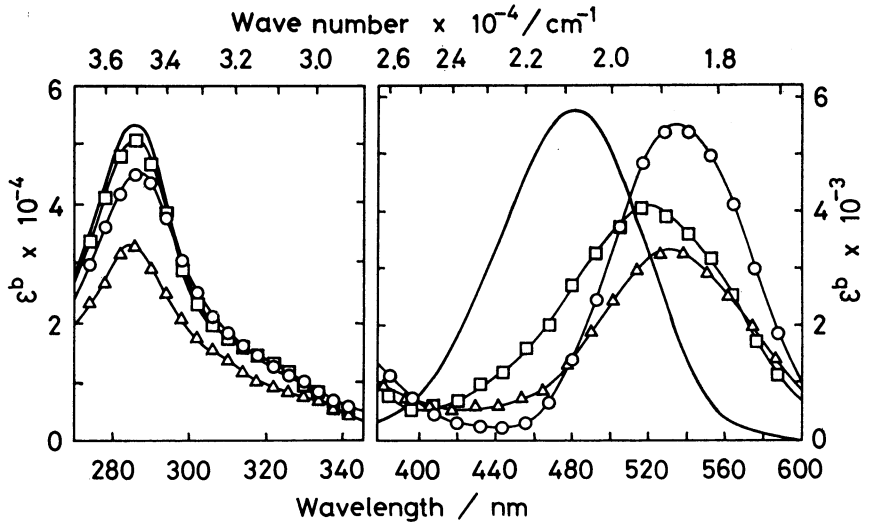

Fig. 4. Visible and ultraviolet spectra of EB bound to three different polyanions. (○) NaPSS, (△) NaPP, and (□) NaPA. The ultraviolet spectra were calculated at $P/D=0.937$ for NaPSS, 0.727 for NaPP, and 1.045 for NaPA. The concentration of EB is 7.47×10^{-5} mol dm^{-3}. Spectra of free EB are shown with solid lines for comparison.

Table 2. Characteristics of bound-EB spectra in the visible wavelength region

Systems	λ^b_{max} [a] / nm	ε^b_{max} [b] / $mol^{-1}\,dm^3\,cm^{-1}$	H[c]	$\Delta\tilde{\nu}_{1/2}$ [d]	F[e]	H'[f]
EB[g]	480	5850	0	3920	0.107	0
EB–NaPSS	536	5550	5.1	2820	0.0751	29.5
EB–NaPP	532	3350	42.7	3410	0.0552	48.3
EB–NaPA	518	4160	28.9	3930	0.0785	26.4
EB–nDNA	524	4190	28.4	2950	0.0613	42.5
EB–dDNA	524	4090	30.1	3030	0.0595	44.2

a) Absorption peak of bound-EB spectrum. b) Molar absorption coefficient of bound EB at λ^b_{max} c) Hypochromicity in % defined as $[1-(\varepsilon^b_{max}/5850)]\times100$. d) Half-intensity band width, where $\varepsilon=\varepsilon^b_{max}/2$. e) Oscillator strength defined as $(4.315\times10^{-9})\int_{1/\lambda_1}^{1/\lambda_2}\varepsilon^b(\tilde{\nu})\cdot d\tilde{\nu}$, where λ_1 is set at 660 nm and λ_2 is the wavelength at which ε^b_λ is minimum. f) Hypochromicity in % defined as $[1-(F_{\text{bound EB}}/F_{\text{free EB}})]\times100$. g) Free EB as the reference.

the series of optical titration spectra were measured for EB–nDNA (isosbestic points 518 and 392 nm) and EB–dDNA (517 and 391 nm) systems. The results of optical titrations are similar to those in Fig. 1 and to those previously reported;[12,18,27] therefore, no detail will be reproduced here. Values of ε_{480} of EB decrease almost monotonously by addition of either native or denatured DNA solution, *i.e.*, the least convex curvature was observed (Fig. 2). The PCA result reveals that the number of EB components responsible for the visible spectra (the DNA moiety does not absorb in this wavelength region) is most probably two in the EB–nDNA and –dDNA systems, as indicated by the relative magnitudes of the eigenvalues in Fig. 3 (d and e). Hence, one free and one bound EB species are present in the low P/D range, regardless of the conformations of DNA.

By the extended PCA procedure, both K_a and α were evaluated (Table 1). Values of α are close to unity, indicating that the frequently-postulated binding scheme K' is nearly applicable to the EB–DNA system. Indeed, the value of K' calculated at $P/D=1$ (and that of K_a) is in good agreement with equilibrium constants previously reported for various EB–nDNA systems.[19,27] Figure 5 shows that the bound-EB spectrum in the native DNA complex (circles) is practically identical with that in the denatured DNA complex (triangles). These spectra are similar to a bound-EB spectrum which was obtained by equilibrium dialysis under different conditions.[19] The bound-EB spectra for DNA are very similar to those for three synthetic polyanions as regards their spectral characteristics, such as the red-shift and the absence of any new metachromasy band (Table 2). This close similarity suggests that the binding site of DNA may be related neither to the overall DNA conformation nor to the presence or absence of base-pairs. The polymer conformations or the functional groups thereof seem to be only of secondary importance for the bound-EB spectra. Thus, the most likely binding site is the charged moiety of the polymer, which is the common factor in the low P/D range.

Because of the reasons stated in the preceding section, the PCA procedure could not be applied to the ultraviolet spectra of EB–nDNA and –dDNA systems. By subtracting the contribution of the moiety of native and denatured DNA, the bound-EB spectra in the ultraviolet region were calculated with values of K_a and α according to Eq. 5 and are shown in Fig. 5. They are again very close to each other and to a reported spectrum.[16]

Fraction of Bound EB and Binding Curves. Values of the fraction of bound EB, f_b, or free EB, $1-f_b$, were calculated with K_a and α in Table 1 or with the elements of the **t** matrix. They are plotted against P/D on a

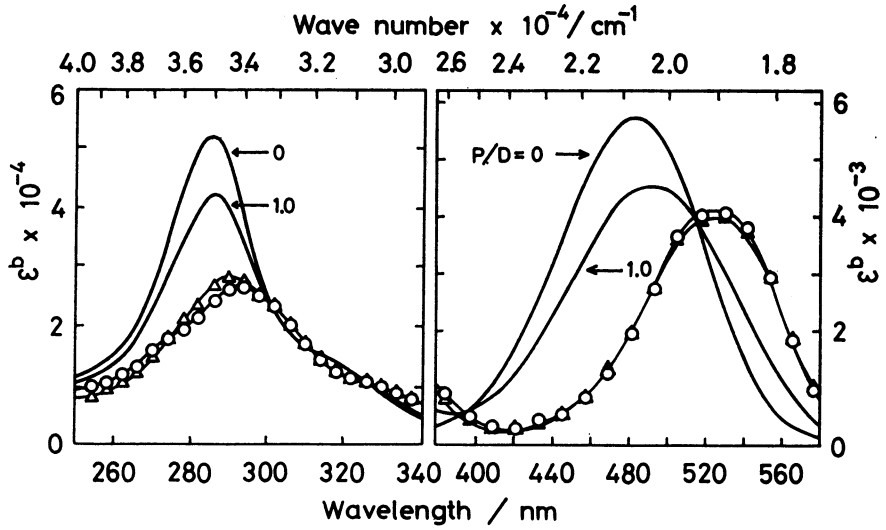

Fig. 5. Visible and ultraviolet absorption spectra of EB titrated with the 1×10^{-3} mol dm^{-3} NaCl-containing DNA solutions and the bound-EB spectra. Observed spectra are at $P/D=0$ and 1.0 for EB–nDNA solutions only. (○) EB bound to native DNA and (△) bound to heat-denatured DNA. The concentration of EB is 7.12×10^{-5} mol dm^{-3} in 1×10^{-3} mol dm^{-3} NaCl.

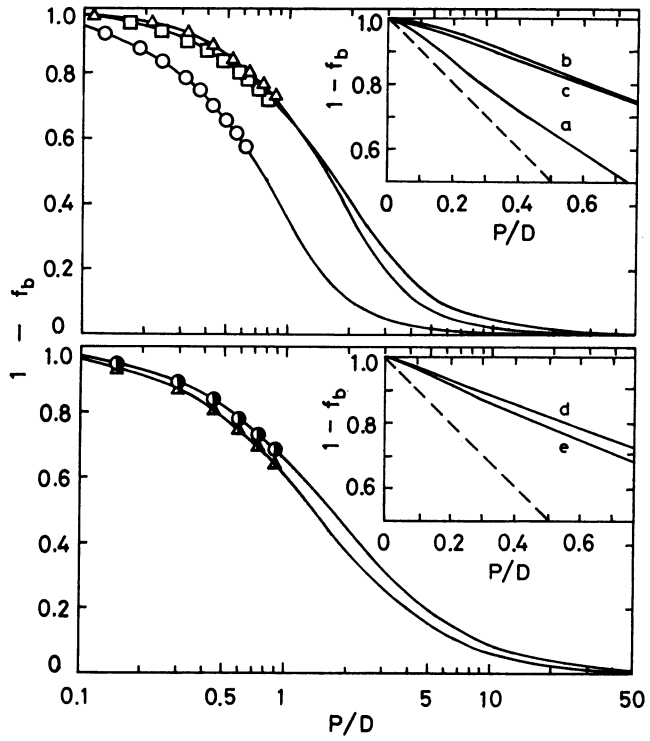

Fig. 6. Fractions of bound-EB in EB–polymer solutions and binding curves as a function of P/D. The ordinates are the fraction of free EB in EB–polymer solution, $1-f_b$. (○) NaPSS, (△) NaPP, (□) NaPA, (◑) nDNA, and (▲) dDNA. Binding curves, calculated with values of K_a and α, are shown by solid lines. In inserts, values of $(1-f_b)$ are plotted against P/D on a decadic scale. Dashed lines should be referred to the text. Each point was calculated with the aid of Eq. 4 in Ref. 4.

semi-logarithmic scale in Fig. 6. The fraction of free EB decreases gradually with the increase in P/D. Almost all EB molecules are estimated to be bound to NaPSS at $P/D>20$ but to NaPA, nDNA, and dDNA only at $P/D>50$, provided that the binding proceeds according to Eq. 1 or 2. The binding affinity of various polymers toward EB expressed by f_b at $P/D=1$ is in the order of NaPSS>dDNA (1 mM NaCl)>nDNA (1 mM NaCl)> NaPP≒NaPA. NaPSS has been noted for its high binding affinity toward various acridine dyes,[3,7] for which the strong ability for counterion condensation may be responsible.[39] Sigmoidal changes of binding curves are well reproduced for EB–polyanion systems ($\alpha>1$), as are indicated in the inserts of Fig. 6, where P/D values are plotted on a decadic scale. The binding curves decrease almost linearly for EB–DNA system ($\alpha=1.1$). In any case, it is worth mentioning that, except for NaPSS, no more than 50% of EB are bound to polymer sites when EB and the polymer residue are present in solution at equimolar amounts ($P/D=1$). Dashed lines in the inserts represent the relation $(1-f_b)=1-(D^b/P)(P/D)$, where $D^b/P=1$, *i.e.*, the number of bound EB per polymer residue in solution is unity. Hence, even in the P/D region where the amount of EB is in large excess ($P/D<1$), polymer sites are only partially occupied by EB (Fig. 6).

Closing Remarks. The common spectral features of five EB–polymer complexes are in that the absorption maximum of each bound-EB spectrum in the visible region shows both bathochromism and hypochromism but none of new "metachromasy" band, band-broadening, or hypsochromism. These features are in accord with those observed for *N*-methylacridinium and 9-aminoacridinium chlorides, but in contrast with the more complex acridine dyes. A possible explanation rests on the fact that the visible absorption band of metachromatic 3,6-disubstituted acridine dyes consists of two mutually perpendicular but overlapping transition moments,[10] while the visible band of EB[35,36] or

simple acridine cations[7,40] consists of a single electronic transition moment. Theoretical interpretations of these spectral characteristics would undoubtedly promote the understanding of metachromasy. For this purpose, accumulations of bound-dye spectra are highly desired for diverse combinations between metachromatic dyes of various chemical structures and polyelectrolytes with different conformations and functional groups.

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