Metachromasy. V.† The Effect of the Chain Length of Polyphosphate on the Metachromatic Behavior of Crystal Violet and Trypaflavine with Emphasis on the Binding Curve and the Spectra of Bound Dyes

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Absorption spectra of two metachromatic dyes, Crystal Violet, CV, and Trypaflavine, TF, were measured in the presence of three sodium polyphosphates, NaPP, of different chain lengths. The optical titrations of these systems were performed at the phosphate residue-to-dye ratios, P/D, between 0: 1 and about 1: 1, where isosbestic points are present in the absorption spectra. The apparent molar absorption coefficients of the two dyes in each system changed sigmoidally with the increase in P/D. The extended principal component analysis was applied to the observed spectra of each dye–NaPP system in order to determine both the equilibrium constant for the binding reaction between the dye and the polymer and the pure spectra of the bound dyes. An empirical parameter α was introduced into the expression for the equilibrium constant, $K=$[complex]/[free dye][unoccupied binding site]$^{\alpha}$, to reproduce the sigmoidal titration curve ($\alpha>1$). The amount of the bound CV and TF increased with the increase in the chain length of NaPP at a given P/D, although the pure spectra of those bound dyes were independent of the chain length for low P/D values. The binding behavior of those dyes for high P/D values was also discussed by comparing the previous data with the binding curves calculated in this work.

The striking change in the absorption spectrum of a metachromatic dye in the presence of polyelectrolyte is generally characteristic of the specific nature of the polymer. For example, such a change has been related to the chain length,[1-3] overall or local conformation,[4-8] and functional or ionizable group of individual polymers.[4,9,10] The interaction between dye and polymer is effected by adjusting the experimental conditions such as pH, temperature, ionic strength, and the molar mixing ratio of polymer residues to dye, P/D. Therefore, detailed studies which take into account these factors quantitatively are necessary for full understanding of both the metachromatic behavior of dyes and the dye–polymer interaction.

The method of extended principal component analysis, the PCA method,[11] was successfully applied to the complicated equilibrium systems of cationic dyes (Crystal Violet, CV, and Trypaflavine, TF) and various polyanions, in order to determine the number of dye species in equilibrium, the equilibrium constants, and the absorption spectra of the bound-dye species.[4] Experimentally, the successive titration method was employed in a series of measurements to ensure the accuracy of the dye concentration within 1%. Since the metachromatic behavior of CV and TF has been shown to depend on the number-average degree of polymerization, $\bar{n}$, of sodium polyphosphate over a wide P/D range of 0—2000,[2,3] the purpose of this article is to apply the extended PCA method to the CV- and TF-NaPP ($\bar{n}=11$, 24, and 64) systems in order to clarify the relationship between the metachromatic behavior of these dyes and the chain length of NaPP.

In this work, the equilibrium constants and the pure absorption spectra of the bound-dye species could be determined for those systems. The optical titration curves of all the systems were sigmoidal within the P/D range from zero to about one. In order to analyze the sigmoidal titration behavior, an equilibrium expression with an empirical parameter α was utilized in the same manner as in the previous work.[4] The present results clearly indicate that the equilibrium constant and the value of α both depend on the combination of dyes and NaPP samples. On the contrary, the pure spectra of the bound-dye species are independent of the chain length of NaPP in the low P/D range. The fraction of the bound dye was calculated against P/D by using the equilibrium constant and the parameter α obtained for each dye–NaPP system. By comparing the binding fraction *vs.* P/D curves with previous data,[2,3] it was concluded that two or more bound-dye species should be present over the entire P/D range. The mechanism of the chain-length dependence on the metachromasy was also discussed quantitatively in terms of the amount of the bound dye and its absorption spectrum. Lastly, the empirical parameter α was qualitatively related to the cooperative parameter q which was defined by Schwarz.[12]

† For the preceding paper of this series, see Ref. 4.

Experimental

Materials. All the NaPP samples ($\bar{n}=11$, 24, and 64) used in this work are the same refractionated preparations as described elsewhere.[2,3] The cationic dyes, CV ($\varepsilon=9.20\times10^4$ at 592 nm) and TF ($\varepsilon=4.67\times10^4$ at 452 nm), are also those used in the previous work.[4]

Procedures and Measurements. A Hitachi EPS-3T recording spectrophotometer was used together with a matched pair of 1 and 2 cm long quartz cells. The temperature was set at 25 °C by circulating regulated water through a cell holder. Procedures of the optical titration and other precautions for measurements were described in Ref. 4. The observed data were analyzed by the extended PCA method, which was described in detail in the previous papers.[4,11]

Results

Absorption Spectra and Optical Titration Curves. The absorption spectra were measured at the different stages of titration with a titrant NaPP solution up to a P/D

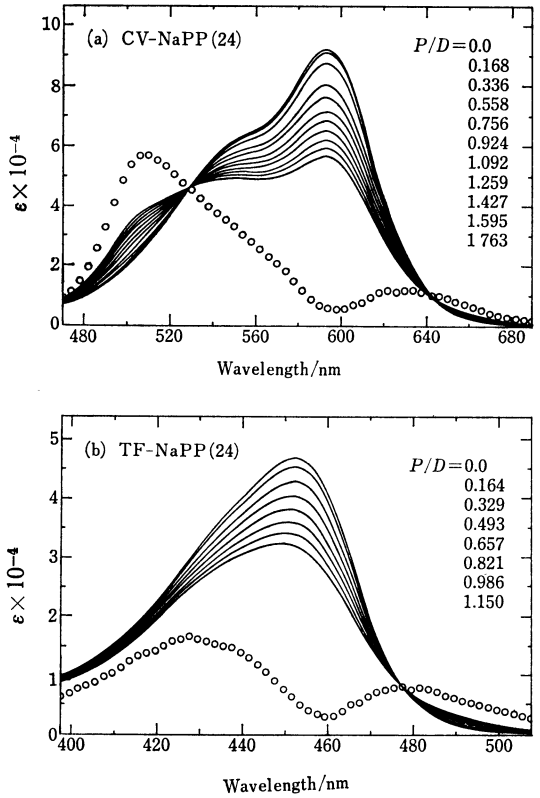

Fig. 1. Absorption spectra of CV (a) and TF (b) in the presence and absence of NaPP(24) and the pure absorption spectra of bound dyes (open circles). The initial concentrations of CV and TF are 8.86 and 11.0 μM (1μM=1×10⁻⁶ mol dm⁻³) respectively. The concentrations of NaPP are given in the respective figures in terms of P/D, the order of which corresponds to the order of the decrease in the maximum absorbance.

value near one. Figures 1(a) and (b) show the absorption spectra of CV and TF in the presence of NaPP whose $\bar{n}$ is 24 (hereafter denoted as NaPP (24)). For other CV- and TF-NaPP (11 and 64) systems, the series of absorption spectra were similarly obtained. Since the observed spectrum of a dye-NaPP solution is a composite of the component spectra of the known free and unknown bound dyes, the apparent molar absorption coefficients, ε, were calculated on the basis of the analytical concentration of the dye in solution. The spectra of CV-NaPP (24) show only a shoulder at 510 nm, as is shown in Fig. 1(a), although the metachromasy band was clearly observed at 510 nm for NaPP(64) and NaPP(154). (The absorption spectra for CV-NaPP(154) and for TF-NaPP(216) and TF-NaPP(154) were shown in Refs. 2 and 4, and Refs. 3 and 4, respectively. They will hereafter be quoted without the references.) In the TF-NaPP(24) system which is shown in Fig. 1(b), the maximum absorption band at 452 nm is shifted gradually with increasing P/D toward the shorter wavelength with no explicit metachromasy band.

Three and two isosbestic points are observed at 460, 529, and 639 nm and at 371 and 476 nm for the CV- and TF-NaPP(11, 24, 64, and 154) systems, respectively. These isosbestic points predict the presence of two absorbing components in each system, free dye and a bound-dye species, between the P/D values of zero and about one. It should also be noted that the ε and position of those isosbestic points remain unchanged regardless of the chain length of NaPP. These findings suggest that the pure spectra of the bound-dye species may not depend on the chain length of NaPP for low P/D values.

The optical titration curves are plotted against P/D at 592 nm for CV and at 450 nm for TF in the presence of NaPP with the varying degree of polymerization in Figs. 2(a) and (b). All the titration curves change sigmoidally, but not monotonously, with P/D in a manner similar to the NaPP(154) systems. The values of the apparent ε at a given P/D (*e.g.*, $P/D=1$) decrease with the increase in the chain length of NaPP for both CV- and TF-NaPP systems.

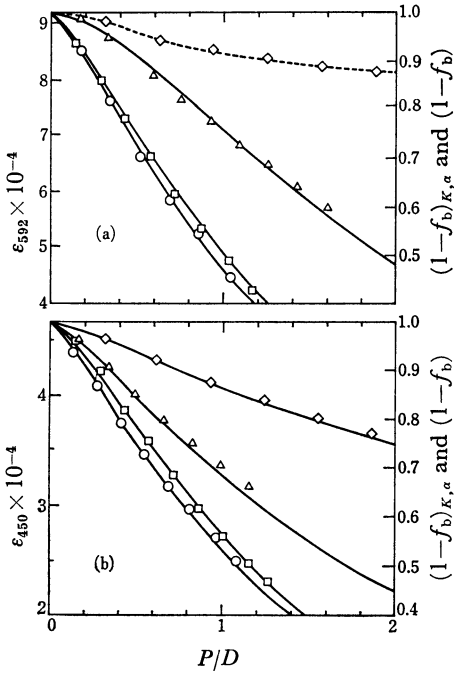

Fig. 2. Optical titration data and calculated binding curves for CV (a) and TF (b). The optical titration data (open symbols) are plotted against P/D in terms of ε_λ at 592 nm for CV and 450 nm for TF (left ordinate). The fractions of free dyes calculated from those data (open symbols) are also indicated in terms of $(1-f_b)$ (right ordinate). □: NaPP(64), △: NaPP (24), ◇: NaPP(11), and ○: NaPP(154). The calculated binding curves (solid lines) are shown in terms of $(1-f_b)_{K,\alpha}$ (right ordinate). The dashed line for the CV-NaPP(11) indicates that the $(1-f_b)_{K,\alpha}$ curve could not be calculated by the PCA method (see text for detail).

Pure Spectra of Bound-dye Species and Equilibrium Constants. The extended PCA method is especially useful for the determination of the equilibrium scheme for binding of dye to polyelectrolyte of relatively low molecular weight which cannot be retained by a semipermeable membrane in equilibrium dialysis.[4,11] Therefore, the absorption spectra of all dye-NaPP systems obtained in this work were analyzed by the same method. The number of the absorbing components was first deter-

mined to be two, free and bound dyes, for all the systems which are expected from the presence of isosbestic points. In order to reproduce the observed sigmoidal and monotonous titration curves, an empirical parameter α was introduced into the equilibrium equation for a dye–NaPP system consisting of such two absorbing components as follows:[4]

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

In this expression D is the free, unbound dye; P means the unoccupied binding site of NaPP; DP* is the bound dye which gives rise to an absorption spectrum different from that of the free dye; the brackets denote the equilibrium concentration. When the value of α is greater than one, the sigmoidal titration curve can be obtained. The pure spectrum of the bound-dye species, equilibrium constant, and empirical parameter were all determined simultaneously from a series of absorption spectra of a particular dye–NaPP system.[4]

Figure 3(a) shows the pure spectra of the CV bound

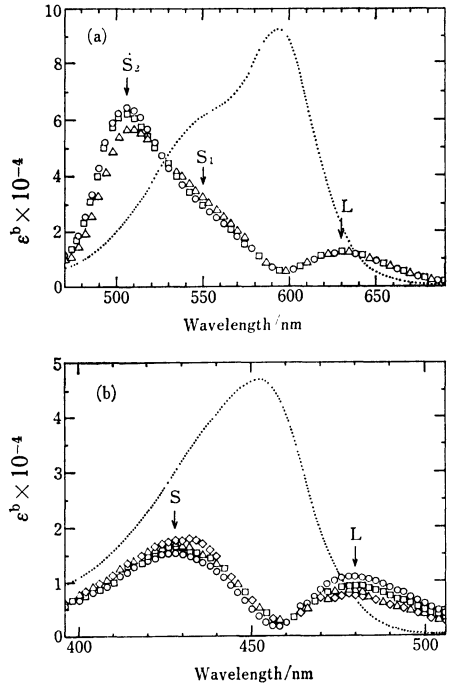

Fig. 3. Pure absorption spectra of CV (a) and TF (b) bound to NaPP's with different chain lengths. □: NaPP(64), △: NaPP(24), ◇: NaPP(11), and ○: NaPP(154). The spectra of free dyes (……) are also shown.

to NaPP(24), NaPP(64), and NaPP(154), thus obtained by the PCA method, together with the free CV for comparison. The pure spectrum of the bound CV for CV–NaPP(11) system could not be obtained from a set of the absorption spectra because their variation was only slight. For all the CV–NaPP systems, the spectra of bound CV exhibit three metachromasy bands: they are designated as the Meta S_2, S_1, and L bands which are located at 506, 550, and 630 nm, respectively, and are indicated by arrows in Fig. 3(a). (The Meta S_1 and S_2 bands are for the short-wavelength ones relative to the peak of the free CV, while the Meta L band is for the long-wavelength one; the numbering is from the one closest to the peak position of the free dye.) The pure spectra of TF bound to NaPP(11), NaPP(24), NaPP(64), and NaPP(154) are shown in Fig. 3(b). They exhibit two metachromasy bands: the Meta S band at 428 nm and the Meta L band at 480 nm. The pure spectra of the bound-dye species show pronounced metachromasy bands (or peaks), in spite of the fact that such bands have not been observed in the spectra for the CV–NaPP(24) or for all of the TF–NaPP systems. Furthermore, the spectral profiles of the bound dyes and the positions of the Meta bands are all independent of the chain length of NaPP bound by either CV and TF. It should be noted that the pure spectra of the bound-dye species have been unraveled in detail by the extended PCA method.

The values of α and of the equilibrium constant K in Eq. 1 were calculated from the series of the absorption spectra of CV- and TF–NaPP systems and are listed in Table 1. It should be noted that the values of α are always greater than one for the sigmoidal titration curves (Figs. 2(a) and (b)). Furthermore, the values of α depend on the combination between dyes and NaPP's with varying $\bar{n}$ values. The binding of dye to NaPP may be affected by the chemical structure of the dye and by the chain length of NaPP with the identical residue structure. Since the chain length may affect the electrostatic potential field of NaPP, the parameter α may be considered as an indicator which reflects the binding mode of dyes. The values of equilibrium constant listed in Table 1 vary widely, because the dimension of K ($=[dm^3\ mol^{-1}]^{\alpha}$) now depends on α. Therefore, the values of K of the different dye–NaPP systems cannot be readily compared with each other to characterize them. However, the equilibrium constant K defined in Eq. 1 may be related to the frequently postulated equilibrium constant K' in such that $K' = [DP^*]/[D][P] = K[P]^{\alpha-1}$. The values of K' were calculated at a representative P/D of 1 and are listed

Table 1. Empirical parameters, α, and equilibrium constants, K and K' at 25 °C for CV- and TF–NaPP systems

$\bar{n}$ of NaPP	CV			TF		
	α	K[a]	K'[b]	α	K[a]	K'[b]
154	1.5	1.0×10^8	2.1×10^5	1.5	5.1×10^7	1.3×10^5
64	1.5	0.98×10^8	2.1×10^5	1.3	7.7×10^6	1.1×10^5
24	1.9	2.2×10^9	0.49×10^5	1.3	2.3×10^6	0.66×10^5
11	—	—	—	1.2	2.1×10^5	0.20×10^5

a) The dimension is given by $[dm^3\ mol^{-1}]^{\alpha}$. b) $K' = K[P]^{\alpha-1}$ at $P/D = 1$.

in Table 1. For both CV and TF, the values of K' increase with the increase in the chain length of NaPP. Thus, the greater part of either dye should be bound to the longer chain of NaPP (*vide infra*).

Binding Curves. The fraction of bound dye, f_b, defined as the ratio of the concentration of bound dye, C_b, to the sum total of the concentrations of the bound and free dyes in solution, C_0, can be calculated at a given P/D in three ways. First, the f_b is obtained with the values of K and α by solving the following equation, which is derived from Eq. 1, for f_b by the Newton method:

$$(1-f_b) = \frac{f_b}{K\{C_0(P/D-f_b)\}^\alpha}. \quad (2)$$

The $(1-f_b)$, *i.e.*, the fraction of free dye, calculated from Eq. 2 is denoted as $(1-f_b)_{K,\alpha}$. The values of $(1-f_b)_{K,\alpha}$ for CV and TF are shown with solid curves in Figs. 2(a) and (b) respectively.

Figures 2(a) and (b) show an excellent agreement between the calculated curve $(1-f_b)_{K,\alpha}$ and the observed points of ε_λ for each dye–NaPP system. Secondly, the fraction of bound dye, f_b, can be calculated from the observed ε_λ at a selected wavelength, according to Lambert-Beer's law, as follows:

$$(1-f_b)_\lambda = \frac{\varepsilon_\lambda - \varepsilon_\lambda{}^b}{\varepsilon_\lambda{}^f - \varepsilon_\lambda{}^b}, \quad (3)$$

where $\varepsilon_\lambda{}^b$ and $\varepsilon_\lambda{}^f$ are the molar absorption coefficients of bound and free dyes at the wavelength λ respectively. Thirdly, the mean value of $(1-f_b)_\lambda$ (*cf.* Eq. 6 in Ref. 4) may be defined as follows:

$$(1-f_b) = \frac{1}{n}\sum_{i=1}^{n}(1-f_b)_{\lambda_i} \quad (n=56), \quad (4)$$

where n denotes the number of selected wavelengths. The $(1-f_b)$ is determined for a given absorption spectrum at a given P/D by the PCA method.[11] The calculated $(1-f_b)_{K,\alpha}$ agrees with the $(1-f_b)$ at all P/D values in Figs. 2(a) and (b). It should be noted that the sigmoidal decrease of the observed ε_λ can be reproduced by $(1-f_b)_{K,\alpha}$ for each system with the value of α larger than one. Hence, the α should be a useful parameter to represent the binding of dye to polyelectrolyte.

The binding curves (solid lines) calculated with the aid of Eq. 2 together with the appropriate α and K in Table 1 are plotted against P/D in Figs. 4(a) and (b). In these figures, the open symbols indicate the points of $(1-f_b)$ which were obtained by analyzing the present optical titration data by the PCA method, while the closed symbols represent the points of $(1-f_b)_\lambda$, $\lambda=592$ and 450 nm for CV and TF respectively, which were calculated from the previous data over a wide P/D range[2,3] with the aid of Eq. 3 together with the values of ε_λ, $\varepsilon_\lambda{}^f$, and $\varepsilon_\lambda{}^b$ (the last was determined in this work by the PCA method). The points of $(1-f_b)_\lambda$ and $(1-f_b)$ obtained from the observed spectra of dye–NaPP fall on the binding curves $(1-f_b)_{K,\alpha}$, which were calculated from Eq. 2, in the P/D range between 0 and 2—3. The $(1-f_b)_{K,\alpha}$ curves approach the limiting value of zero, as the P/D values further increase.

On the contrary, the points of $(1-f_b)_\lambda$ deviate from the $(1-f_b)_{K,\alpha}$ curves and gradually ascend back to the original value of unity[2,3] passing through the minima in the P/D ranges of 2—8 for CV–NaPP and 3—10 for TF–NaPP. (The P/D values for the minima slightly depend on the chain length of NaPP). In addition, the isosbestic points remaining at the low P/D values of less than *ca.* 2 tend to disappear gradually in those intermediate P/D ranges. Both the deviation of the $(1-f_b)_\lambda$ points from the calculated binding curve (solid line) and the concurrent disappearance of the isosbestic points strongly indicate that some unknown bound-dye species are being formed in the dye–NaPP solution with the increase in P/D. These results also eliminate the possibility that the bound dye begins to dissociate from the polymer site. (If this were the case, the isosbestic points should remain.) Thus, it is most reasonable to conclude that two or more of the bound-dye species are present in the dye–NaPP solution in the intermediate P/D range 2—30. Since the pure spectrum of one of them has already been determined in the previous section (Fig. 3), it is worth now finding out the pure spectrum of the other bound-dye species.

At present, the pure spectrum of the unknown bound-dye species cannot be determined uniquely, because both the number of the bound-dye species and the fraction of each bound-dye species are not available yet in the intermediate P/D range. The sum total of the bound-dye species, however, can be estimated from the calculated binding curve. Therefore, the apparent spectrum of the mixture of bound-dye species, *i.e.*, the mixed spectrum of the bound dyes, may be calculated by using the f_b value, which is obtained from the calculated binding curve, $(1-f_b)_{K,\alpha}$, (solid line in Fig. 4), and the observed spectrum of the dye–NaPP solution, ε_λ, at a given P/D value as follows:

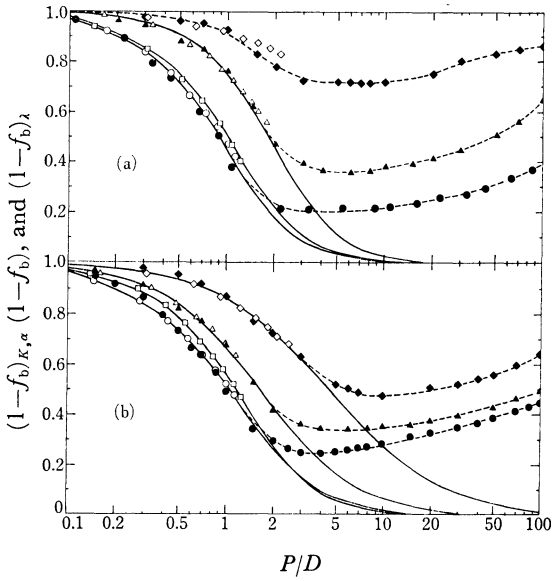

Fig. 4. Binding curves of CV (a) and TF (b) to NaPP's with different chain lengths. The binding curves were calculated in three ways: solid lines for $(1-f_b)_{K,\alpha}$, open symbols for $(1-f_b)$; and closed symbols with dashed lines for $(1-f_b)_\lambda$ ($\lambda=592$ nm for CV and 450 nm for TF). For details, see text. □: NaPP(64), △: NaPP-(24), ◇: NaPP(11), and ○: NaPP(154) for CV and NaPP(216) for TF.

$$\varepsilon_\lambda^{mb} = \varepsilon_\lambda^{f} + \frac{\varepsilon_\lambda - \varepsilon_\lambda^{f}}{f_b} \quad (5)$$

where ε_λ^{mb} is the apparent molar absorption coefficient of the mixture of bound-dye species at a wavelength of λ. For each dye–NaPP system, the mixed spectrum of bound dyes, ε_λ^{mb}, was calculated at the minimum point of the observed $(1-f_b)_\lambda$ *vs.* P/D curve (dashed line in Fig. 4), where no isosbestic point exists. The results are shown for the bound CV and TF in Figs. 5(a) and (b), respectively. Figure 5 shows that the separation between the Meta S and L bands of the mixed spectrum of multiple bound-dye species is apparently narrower than that of the corresponding pure spectrum of a single bound-dye species obtained in the low P/D range (*cf.* Fig. 3), and that the values of ε_λ^{mb} are generally greater than those of ε_λ^{b} in the wavelengths where the peak of the spectrum of free dye is located.

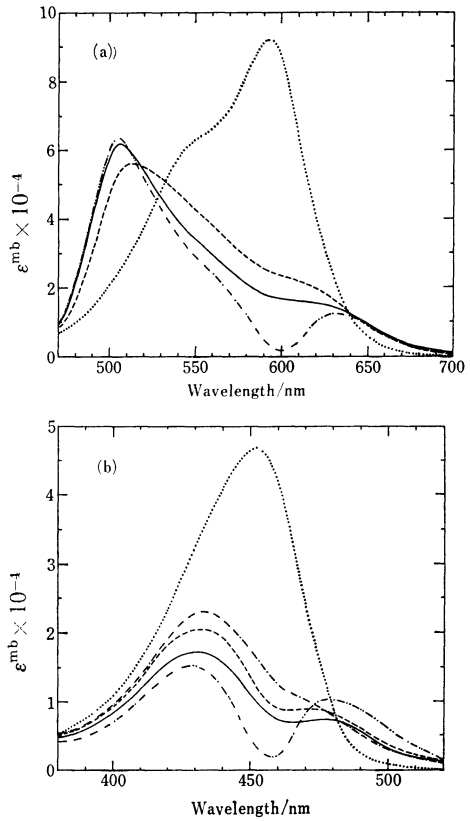

Fig. 5. The mixed bound-spectra of CV (a) and TF (b) calculated at the minimum values of P/D in the $(1-f_b)_\lambda$ *vs.* P/D curves in Fig. 4. CV–NaPP(154) and TF–NaPP(216) at $P/D=3$ (——), CV- and TF-NaPP(24) at $P/D=4$ (----), and CV- and TF-NaPP(11) at $P/D=10$ (-·-·-). Pure absorption spectra of a single bound-dye species obtained in the low P/D region (-···-···-) and the respective free dyes (·····) are also shown.

Discussion

Absorption Spectra of Bound CV and TF. The peaks or shoulders of the pure spectra of the bound CV or TF (at 506, 550, and 630 nm for CV, and at 428 and 480 nm for TF) are independent of the chain length of NaPP (Figs. 3(a) and (b)). On the other hand, the values of ε_λ^{b} for the metachromasy bands vary slightly with the chain length of the NaPP to which CV or TF is bound. However, their difference is probably due to the experimental errors, which include the readout of a series of overlapping spectra.[11] Hence, together with the fact that the isosbestic points exist at the constant wavelengths for all the families of the absorption spectra of either CV or TF in the presence of NaPP with the varying value of $\bar{n}$, the spectrum of bound CV or TF can now be concluded to be independent of the chain length of the NaPP samples ($\bar{n}=11$—154) in the low P/D range of 0—*ca.* 2. This conclusion excludes, at least in the low P/D range, one of two possible hypotheses: that the "flexibility" of the polymer backbone, to which dyes are bound, is responsible for the origin of metachromasy.[2]

Shirai *et al.*[9] investigated the metachromasy of Methylene Blue in the presence of potassium poly(ethylene sulfate)s which were different in the degree of sulfation. The metachromasy band of Methylene Blue shifted to the longer wavelength region with the decrease in the degree of sulfation of the polyanion. In the previous report,[4] the position of the metachromasy band was shown to vary considerably with the conformation of polyanion, even if their residue structures are identical or closely related. This situation is especially remarkable in the dye–NaPP and –DNA systems in which the peak positions of the metachromasy bands of bound CV (and also TF) are different, although both NaPP and DNA have the ionized phosphate residues as the binding site for those dyes. On the other hand, the positions of the metachromasy bands of bound CV and TF do not vary with the chain length of NaPP's, each of which has the identical backbone structure and binding site for dyes (Fig. 3). These results all lead to the conclusion that the peak positions of the metachromasy bands of a metachromatic dye depend on the local conformation of the binding site of polymers. It is also reasonable to conclude that the configuration of the π electrons of the bound dye may be affected by the mean distance between the binding sites of a particular polyanion.

Empirical Parameter α and Sigmoidal Titration Curve. As shown in Figs. 2(a) and (b), the ε_λ and $(1-f_b)_{\kappa,\alpha}$ decrease sigmoidally with the increase in P/D from zero to about one in all cases. The empirical parameter α was introduced to reproduce a sigmoidal or monotonous titration curve.[4] Schwarz[12] proposed a theory for the binding of a dye to a linear lattice in which cooperative interaction is restricted to the nearest neighbor binding sites. Subsequently, Schwarz *et al.*[15,16] applied the theory to the monotonous titration curves of some Proflavine-polyanion systems and determined the cooperative binding constant and the parameters, g (the number of the binding sites per segment of polymer) and q (a factor measuring the strength of cooperativity). However, it is obvious that the sigmoidal titration curve cannot be reproduced by using those parameters q and g, which are assumed to be constant for a given dye–polyanion system. The sigmoidal titration curve means that the binding of dye is inhibited near the limiting P/D value ($P/D=0$), and gradually

accelerated with the increase in P/D, whereas a monotonous one means that the binding of dye is either independent of P/D or retarded with the increase in P/D. In Schwarz's theory, the titration curves with $q>1$ indicate positive cooperativity, while those with $q<1$ indicate negative cooperativity. Therefore, the following qualitative relations may be summarized between the empirical parameter α and Schwarz's cooperative parameter q: (i) for $\alpha>1$ (sigmoidal), the parameter q increases continuously with increasing P/D, which contradicts Schwarz's assumption that q is a constant; (ii) for $\alpha=1$ (monotonous), the q is constant; and (iii) for $\alpha<1$ (monotonous), the q decreases continuously with increasing P/D, which also contradicts his assumption.

Binding Curves. The binding curves (solid lines) shown in Figs. 2(a) and (b) reveal that the fraction of the bound dye increases at a low P/D value with the increase in the chain length of NaPP. On the other hand, the spectra of bound CV and TF are independent of the chain length of NaPP in the same low P/D range ($P/D<ca.$ 2) where a single bound-dye species exists. Since the ε_λ is related to the fraction of bound dye by Eq. 3, the dependence of the observed metachromasy on the chain length of NaPP in the low P/D range can be attributed to the difference in the fraction of CV (or TF) bound to the NaPP with different chain lengths. This view is supported from the fact that the $(1-f_b)_{\kappa,\alpha}$ *vs.* P/D curves coincide with the points of various symbols in Figs 4(a) and (b).

The result that the fraction of bound CV and TF varies with the chain length of NaPP is an interesting problem. Schindewolf[13] reported that the degree of dissociation of the Na^+ counter-ion from NaPP decreases sharply with the increase in the chain length in aqueous solution. Kielman and Leyte[14] measured the longitudinal relaxation time, T_1, of the $^{23}Na^+$ ion in NaPP solution by NMR. Since the value of $(T_1)^{-1}$ can be related to the amount of the bound and free Na^+ ions, its sharp increase clearly indicates that the amount of the bound Na^+ ions increases with the increasing chain length of NaPP up to $\bar{n}\approx 60$. Two alternative views on the binding mechanism of a dye to NaPP are considered below.

The first view is that the dye competes with the Na^+ ion upon binding to NaPP. The aforementioned results suggest that larger amounts of both the dye and Na^+ ion are bound to the NaPP with the longer chain length. If both the dye and Na^+ ion are bound to NaPP by the electrostatic force, and if the force becomes stronger with the increase in the chain length of NaPP, the dye would compete with the Na^+ ion in binding to the ionized group of NaPP in the low P/D range. In order to examine the competitive binding between the dye and Na^+ ion to NaPP, the concentration of the free counter-ion has to be measured in the presence of dye. The second view is that the counter-ion Na^+ participates in the formation of a dye–polymer site complex directly, as is described by Eq. 13 of Ref. 4, or indirectly, as is indicated by Eq. 1 in this work. The direct participation of the Na^+ ion in the binding between a cationic dye and an ionized phosphate residue of NaPP may be examined by means of electrophoresis of the dye–NaPP solution. On the other hand, the indirect participation of the Na^+ ion (*e.g.*, the charge neutralization of the nearest neighbor phosphate residue) may be studied by the potentiometric[17] and conductometric[2,3] measurements. Certainly these are the interesting subjects to be investigated in detail.

The experimental points (various symbols) in Figs. 4(a) and (b) begin to deviate from the calculated binding curves (solid lines), as the P/D value reaches about 2 to 3. When the curves approach zero, the points ascend again in the higher P/D range, as if the dye were dissociating from NaPP. Concurrently the isosbestic points disappear in the absorption spectra as the P/D becomes larger than about 2—3; that is, there are three or more dye species in a dye–NaPP solution. The ultrafiltration of TF–NaPP(216) has verified that TF is still bound to NaPP at a high P/D value of 1000.[3] Furthermore, the new isosbestic points appear at 636 nm for CV and at 374 and 479 nm for TF in the P/D range higher than *ca.* 30. Thus, it is concluded that there are probably two kinds of bound-dye species in this P/D range. As a consequence, the experimental binding curves (dashed lines) may be classified into three P/D ranges: (i) the low P/D range of 0—near 2 where free dye and only one type of the bound-dye species are present, (ii) the intermediate P/D range of 2—30 where the free and two or more types of bound-dye species are present, and (iii) the high P/D range where two or more types of bound-dye species are present probably without the free dye.

The change of the absorption spectrum with P/D in the high P/D range may be due to the redistribution of the bound-dye species on polymer sites as the sites increase relative to the bound dye. Although many authors[12,18-20] have treated statistically the redistribution of bound dyes on a polyelectrolyte chain assuming an infinitely long chain length, there remain many questions to be answered, *e.g.*, the number and absorption spectra of bound-dye species and the scheme for the redistribution of the bound-dye species on a single (or multiple) polymer chain of finite length. The application of the extended PCA method to the precise experimental data covering a wide P/D range will shed light on these problems.

The Mixed Bound-spectrum. The mixed spectrum of bound-dye species (Figs. 5(a) and (b)) may be composed of two or more absorbing components, one of which is present in the low P/D range. The bound-dye species newly appearing in the intermediate and high P/D ranges should show an absorption spectrum in which the separation between the Meta S and L bands is small. If the energy separation between the two bands is directly proportional to the perturbation of the π electronic system of bound CV or TF resulting from the interaction between the dye and the polymer site or between the bound dyes themselves, such perturbation may be smaller in the intermediate and high P/D ranges than in the low P/D range possibly because of the redistribution of the bound dyes on the polymer.

From the fact that the pure spectra of a single CV or TF species in the low P/D range are independent

of the chain lengths of NaPP (Fig. 3), the difference in the mixed spectra of the multiple CV or TF species (Fig. 5) should result from the polyelectrolyte property of NaPP. In other words, the chain lengths of those NaPP samples ($\bar{n}$=216, 154, 64, 24, and 11) may be responsible for the formation of the bound dye species with the varying types upon the possible redistribution on the polymer sites with the increase in P/D. The effect of the chain length of NaPP on the metachromatic behavior of CV and TF[2,3] is now confirmed quantitatively to originate from a complicated interplay between the free dye and two or more bound-dye species. That is, the pronounced changes in the ε_λ *vs.* P/D curves given in Figs. 3 and 5 of Ref. 1, in Fig. 3 of Ref. 2, and in Fig. 3 of Ref. 3, and the $(1-f_b)_\lambda$ *vs.* P/D curves in Fig. 4 of this work should result from the variation of the relative amounts not only between free and a single bound-dye species (P/D=0—*ca.* 2) but also between multicomponent bound-dye species ($P/D \geq 2$) themselves. Since the extended PCA method is powerful in the multicomponent system,[4] its application to the series of the absorption spectra of the metachromatic dye–polyelectrolyte system over an entire P/D range should yield information leading to the fuller understanding of metachromasy.

Conclusion

The dependence of the chain length of NaPP on metachromasy of CV and TF was quantitatively clarified for the first time. The equilibrium constant, an empirical parameter, α, and the pure absorption spectra of the bound CV and TF species were determined by the PCA method. Both the equilibrium constant and the value of α depend on the chain length of NaPP, while the pure spectra of bound CV and TF are independent of it. Two or more of the bound-dye species should exist over the entire P/D range. From the profiles of the mixed spectra of the multiple bound-dye species, the effect of the chain length of NaPP on metachromasy was concluded to be such that the redistribution of the bound dyes would be promoted with the increase in the chain length.

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