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Metachromasy. III.* Metachromatic Behavior of Trypaflavine in the Presence of Polyphosphates with Various Chain Lengths

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The interaction of trypaflavine with sodium polyphosphate in aqueous solutions in the pH range 6—7 has been investigated by means of light absorption spectroscopy, conductometric titration and ultrafiltration. The metachromatic behavior of the bound dye was characterized in terms of hypso- and hypochromic effects in both visible and UV regions where no pronounced metachromasy band was observed. The effects were studied by varying the mixing ratio of polymer residue to dye, P/D, from 0.1 to 10000. They were found to be maximum in the P/D range 2–10. The effect of the chain length of polyphosphate on metachromasy was determined by use of fourteen refractionated samples with the degree of polymerization, $\bar{n}$, ranging from 8 to 158. No metachromasy appeared in samples with $\bar{n}<7$. It fully developed for those with $\bar{n}>20$, sharply depending on the chain length between 7 and 20. By conductometric titration and ultrafiltration, it was verified that the dye binds to the ionized phosphate almost at 1 : 1, and that the disappearance of metachromasy with the increase in P/D values or with the addition of salts results from two entirely different processes. The results were compared with the data for other dyes.

The physicochemical basis of metachromasy still remains to be clarified because of its extraordinary complexity.[1,2] However, it has become apparent that the metachromatic behavior of cationic dyes depends on the combination of the chemical structure of an individual dye with the conformation of a given polyelectrolyte having a specific functional group (binding site).[1,3,4] Studies of metachromasy have been directed to the interaction between biopolymers, DNA and RNA in particular, and various kinds of dyes whose structures are closely related, because of the practical application to selective staining of nucleic acids.[5–8] Such interaction is complicated due to the intricate structure of the biopolymers. Thus, in order to understand the mechanism involved in metachromasy, certain simpler polymeric models, one of which is polyphosphate, have been used.[9–12]

Metachromatic change of crystal violet (CV) occurs in the presence of sodium polyphosphate (NaPP).[1,2] The present work was undertaken with two objectives; to see whether the previous findings are confined to CV, a triphenylmethane dye, or whether they can also be observed with dyes of another class, such as cationic acridines, and also to provide basic data for a study on competitive binding to polyphosphate between two dyes of different classes.

As an acridine dye, trypaflavine (TF)—3,6-diamino-10-methylacridinium chloride—was chosen for the following reasons. The metachromatic behavior is close to that of proflavine — 3,6-diaminoacridinium chloride — but differs from that of acridine orange and its homologs, all of which have been known to interact with DNA.[3,13–15] Both TF and proflavine have been suggested to constitute a subgroup among the symmetrical 3,6-disubstituted acridines.[3,8] TF was also used together with CV in a study on competitive binding to DNA.[16] From the results obtained by means of absorption spectroscopy, conductometric titration and ultrafiltration, the metachromatic behavior of the NaPP–TF system has been compared with that of the NaPP–other related dye systems.

Experimental

Materials.　All NaPP samples are the same refractionated preparations as reported,[2] unless otherwise stated. TF in the chloride form (Chroma Gesellschaft, Schmidt & Co.) was used without further purification. The maximum molar extinction coefficient, ε, at 451 nm was found to be 42000 at 1.19×10^{-5} M.

Procedures and Measurements.　Procedure for the preparation of stock solutions and polymer–dye mixtures has been reported.[2] The final concentration of TF was kept at 1.19×10^{-5} M, unless otherwise specified. Measurements of the absorption spectra and electric conductivity of NaPP–dye solutions were carried out with the precautions described previously.[2]

Ultrafiltration.　In order to separate the unbound dye from the bound one in the NaPP–dye solution, a Millipore Sweenex ultrafiltration cell was used with the PSAC membrane which nominally retains molecular weights higher than 1000. Since the complete retention of a high molecular weight NaPP sample ($\bar{n}=216$) by the membrane was confirmed, this sample was used for the filtration experiments.

Results

UV and Visible Spectra of TF in the Presence of NaPP in Aqueous Solutions.　The UV and visible absorption spectra of TF in the presence of a high molecular weight NaPP ($\bar{n}=216$) are shown in Fig. 1. The value of P/D as defined previously[2,17] was varied by adjusting the polymer concentration at a constant concentration of TF. The absorption maximum of TF at 451 nm was affected hypso- and hypochromically by the addition of NaPP up to P/D about unity, while the family of spectra showed two isosbestic points at 475 nm and 370 nm. The metachromatic behavior of the absorption maximum at 262.5 nm was similar to that of the 451 nm band, the isosbestic point being at 304 nm. As the P/D value increased from unity to approximately 3, the hypso- and hypochromic effects were enhanced in both UV and visible regions, no isosbestic point being observed. With the further increase in P/D value, the spectral trends reversed very slowly, three isosbestic points appearing

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[*] Part II, Ref. 2.

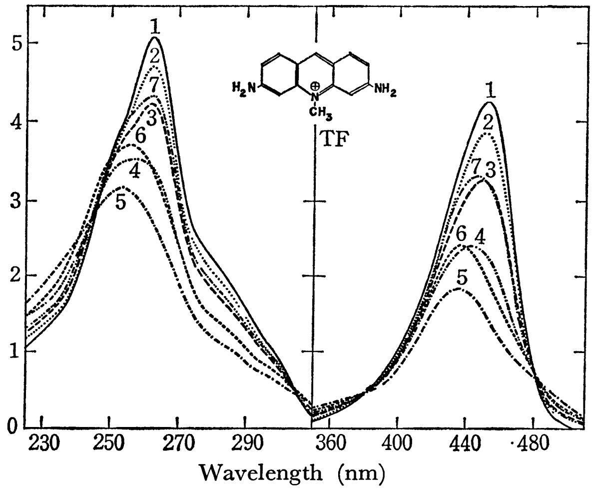

Fig. 1. Visible and UV spectra of TF in the presence of NaPP ($\bar{n}$=216) at various P/D values in aqueous solutions.

(1) pure TF (P/D=0) for comparison, (2) P/D=0.3, (3) P/D=0.5, (4) P/D=1.0, (5) P/D=3.0, (6) P/D =50, (7) P/D=850.

at 479, 374, and 307 nm. At exceedingly high P/D values, the overall spectra became almost the same as the spectrum of TF alone. It should be noted that, in contrast with the data on the NaPP–CV system,[1,2] no pronounced metachromasy band was observed for TF.

The effect of the chain length of NaPP on the UV and visible spectra of the NaPP–TF system is shown in Fig. 2. The P/D value was held at 3 (most appropriate for the metachromatic change of TF). The spectra are essentially the same as the spectrum of TF alone when the number-average degree of polymerization, $\bar{n}$, is less than 5. With an increase in the $\bar{n}$ of NaPP, the band intensity at 451 and 262.5 nm decreased, while the peak positions shifted towards the shorter wavelength. The decrease in intensity occurred in a narrow region of $\bar{n}$ approximately between 7 and 20. Since the dependence of the metachromatic behavior of TF on P/D and $\bar{n}$ is characterized by hypso- and hypochromic effects, all the results are hereafter expressed in terms of both the molar extinction coefficients at 451 nm, ε_{451}, and at 262.5 nm, $\varepsilon_{262.5}$, and the wavelength shift from either the 451 or 262.5 nm maximum of TF, $\Delta\lambda=\lambda(\varepsilon_{\max})-451$ or 262.5 nm. The results of the prominent shoulder at 285 nm are not included, because its change is similar to that of the 262.5 nm band.

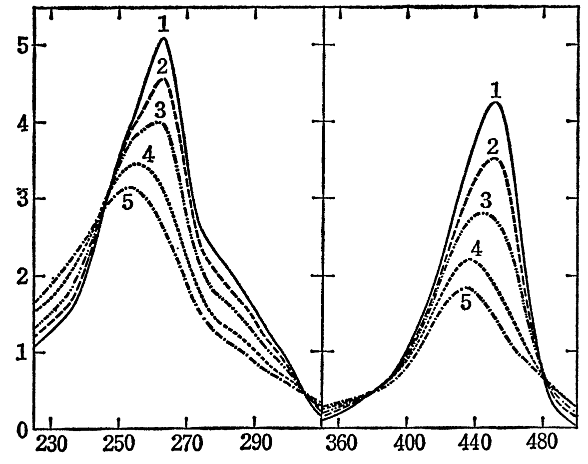

Fig. 2. Visible and UV spectra of TF in the presence of NaPP with various chain lengths at a P/D value of 3 in aqueous solutions.

(1) pure TF (P/D=0) for comparison, (2) $\bar{n}$=8, (3) $\bar{n}$=11, (4) $\bar{n}$=23, (5) ^{n}=158.

Effect of the P/D on the Absorption of TF in the Presence of NaPP with Various Chain Lengths. In Figs. 3a and 3b, the molar extinction coefficient (open) and

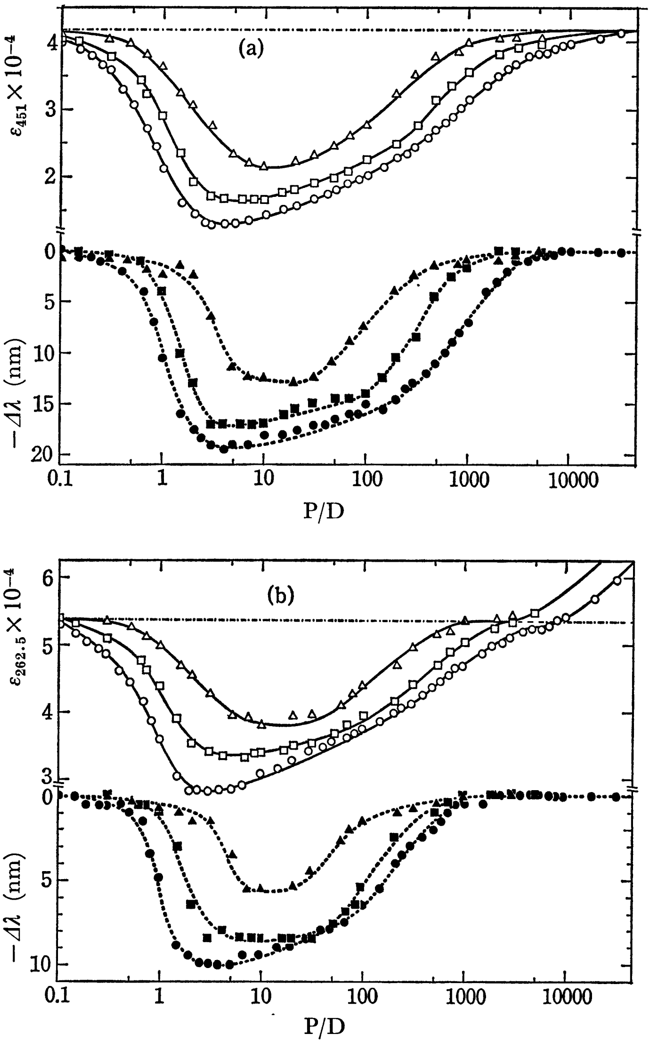

Fig. 3. The dependence of the hypo- and hypsochromisms of TF on the P/D value in the presence of NaPP with different chain lengths in the (a) visible and (b) UV regions.

Open symbols are the molar extinction coefficients of NaPP-TF at (a) 451 nm and (b) 262.5 nm (horizontal lines are those of TF alone), and filled ones are the wavelength shifts of the absorption maximum from (a) 451 nm and (b) 262.5 nm, defined as $\Delta\lambda\equiv\lambda(\varepsilon_{\max})-451$ and 262.5 nm, respectively. -○- and -●- for $\bar{n}$=216, -□- and -■- for $\bar{n}$=24, -△- and -▲- for $\bar{n}$=11.

wavelength shift (filled) of TF are plotted against P/D in the presence of three NaPP samples which give rise to a small ($\bar{n}=11$), medium ($\bar{n}=24$), and large ($\bar{n}=216$) metachromatic change, respectively.[2] The intensity of the 451 and 262.5 nm bands became minimum and the corresponding wavelength shift maximum at a P/D value of approximately 7, 3, or 2 for each NaPP–TF system ($\bar{n}=11$, 24, or 216, respectively). The minimum value of ε at this P/D value depends on the chain length of the polymer.

The ε and band position of the NaPP–TF system approach those of TF alone at both extremely low and high values of P/D** (Figs. 3a and 3b). In order to see if the approach results from the dissociation of TF from the polymer site, filtration of an aqueous solution of TF in the presence of NaPP ($\bar{n}=216$) at a high P/D value of 1000, was carried out (*cf.* Experimental part). Three filtrates (3.0 ml each) were successively collected from the solution (total 15 ml). No filtrates showed the coloration of TF. In contrast, the filtrates from a NaPP–TF solution at a low P/D value of 0.6 all showed the spectrum of pure TF. It is obvious that TF is bound to the polymer site at very high P/D but partly liberated from it at low P/D.

Effect of the Chain Length of NaPP on the Absorption of NaPP–TF Solutions at Various Values of P/D. The effect of the chain length of NaPP on the metachromatic change of the NaPP–TF system is shown in Fig. 4, where the molar extinction coefficient and band shift are plotted against the chain length of NaPP. The P/D value chosen here is each responsible for a large (P/D=3), medium (P/D=30), and small (P/D=300) metachromatic change. The band intensity decrease and peak position shift toward the shorter wavelength occur in a narrow range of $\bar{n}$ between 7 and 20. Both ε and $\Delta\lambda$ leveled off, as the chain lengths of NaPP approach *ca.* 100 and over. The midpoints of those sigmoidal changes for the visible band are located at about $\bar{n}=10$ and 11 (P/D=3), $\bar{n}=8$ and 9 (P/D=30), and $\bar{n}=11$ and 19 (P/D=300), respectively (Fig. 4a). Similar changes are also noted for the UV band (Fig. 4b). It is evident that the metachromatic behavior of the TF bound to NaPP is affected by the chain length of the polymeric moiety and that the critical chain length is associated with oligomeric dimensions.

Influence of Small Ions on Metachromatic Changes. The small ions affect visible and UV bands of TF bound to NaPP ($\bar{n}=154$ and 24) at (a) P/D=3 and (b) P/D=30 (Figs. 5a and 5b). At each P/D value, the effect of the addition of sodium phosphates on the absorption spectra was negligible when the salt concentration was comparable with that of TF (10^{-5}—10^{-4} M). As the salt concentration was increased to 1×10^{-3} M, however, the effect became apparent: both ε and $\Delta\lambda$ changed hyper- and bathochromically relative to those of the low salt solution. At the exceedingly high salt concentration of the order of 10^{-2} M (about 1000 Na^+ ions per dye), the whole spectrum of the NaPP–TF solution was reduced to the spectrum of TF alone. In order to see if the dye is dissociated from the polymer site in the presence of the salt, ultrafiltration of an NaPP($\bar{n}=216$)–TF solution at the salt concentration and P/D of 1×10^{-2} M and 30 was carried out. Three colored filtrates (3.0 ml each) were successively collected from the NaPP–TF solution (16 ml initially). The visible spectrum of each filtrate was identical with that of free dye and that of the initial NaPP–TF solution within experimental error. The bound TF is evidently compelled to dissociate from the site in the presence of an excess of the small ions. In Figs. 5a and 5b, the midpoints of the sigmoidal changes of ε and $\Delta\lambda$ for the longer NaPP–TF solutions are displaced towards the higher salt concentrations. This indicates that more small ions are needed to dissociate the bound TF from the NaPP with a longer chain length.

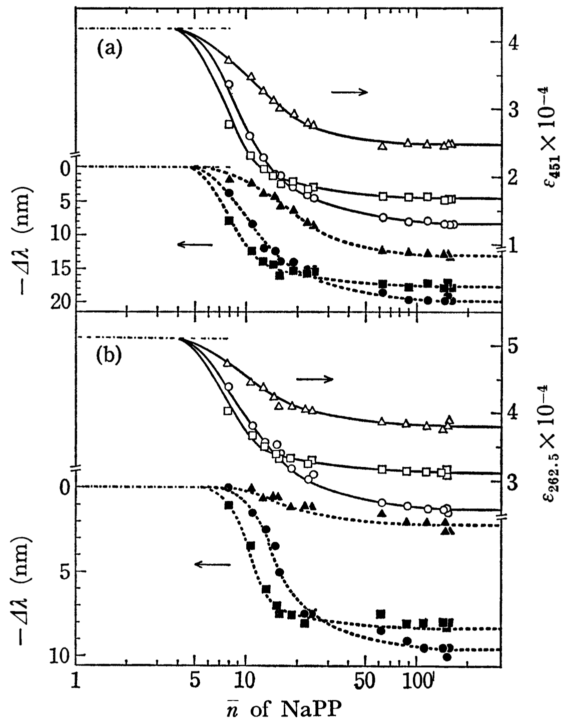

Fig. 4. The dependence of the hypo- and hypsochromisms of the NaPP–TF system on the chain lengths of NaPP at various P/D values in the (a) visible and (b) UV regions.

Open symbols are the molar extinction coefficients of NaPP–TF at (a) 451 nm and (b) 262.5 nm, and filled ones are the wavelength shifts of the absorption maximum in the presence of NaPP from (a) 451 nm and (b) 262.5 nm as defined in Fig. 3. -○- and -●- for P/D=3, -□- and -■- for P/D=30, -△- and -▲- for P/D=300.

Conductometric Titration of TF with NaPP in Aqueous Solutions. The results of conductometric titration of TF in aqueous solutions with three NaPP samples ($\bar{n}=11$, 24, and 154) are shown in Figs. 6a—6c. In the absence of TF the conductivity of the NaPP solution is proportional to the polymer concentration regardless of the chain length, although

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** The $\varepsilon_{262.5}$ value, but not the band shift for the 262.5 nm band, exceeds that of TF at extremely high P/D. This is because polyphosphate absorbs light in this region.[18]

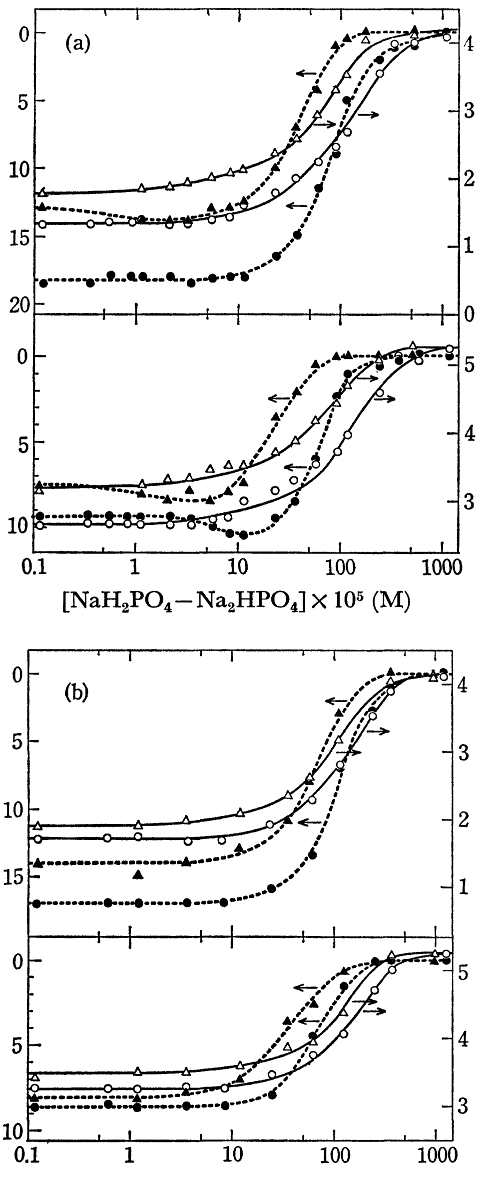

Fig. 5. The effect of sodium phosphates on the hypo- and hypsochromisms of TF in the presence of NaPP at two P/D values (a) P/D=3 and (b) P/D=30. Open symbols are the molar extinction coefficients of NaPP-TF at 451 nm (upper half) and 262.5 nm (lower half), and filled ones are the wavelength shifts of the absorption maximum from 451 nm (upper half) and 262.5 nm (lower half). -○- and -●- for NaPP with $\bar{n}=154$, -△- and -▲- for NaPP with $\bar{n}=24$.

the slope is less steep for the higher $\bar{n}$ sample. In the presence of TF, the conductivity of the titrated solution also increases linearly. However, a break occurs at a P/D value of about unity (1.2, 1.1, and 1.0) for each NaPP sample ($\bar{n}$=11, 24, and 154) as indicated by the intersection of two interpolated lines. Considering the fact that the deviation of the observed points from linearity results from the equilibrium between bound and unbound TF, the conductometric data seem to indicate that TF could be bound to the

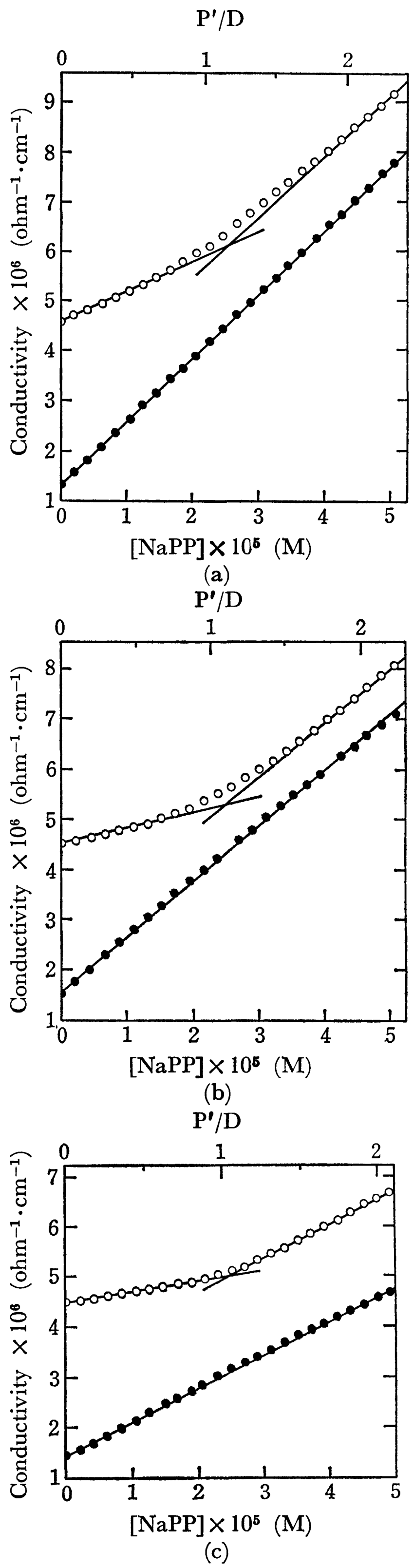

Fig. 6. Conductometric titration of TF with NaPP with various chain lengths (a) $\bar{n}$=11, (b) $\bar{n}$=24, and (c) $\bar{n}$=154.

-○- for the dye solution (the initial concentration of TF 2.26×10^{-5} M) titrated with NaPP and -●- for the blank solution titrated with NaPP for comparison. The concentration of NaPP is corrected for volume increase. P′ is the concentration of the ionized group of the polymer and corrected as equal to $P(1+1/\bar{n})$ where P is the residue concentration.

ionized phosphate residue almost up to a one-to-one ratio regardless of the polymer chain length.

Discussion

Comparison of Metachromatic Behavior between TF and CV in the Presence of NaPP. The metachromatic behavior of the 451 nm band of the NaPP–TF system (Fig. 1) considerably differs qualitatively from that of the 592 nm band of the NaPP–CV system,[2] although many quantitative aspects are common to both TF and CV. The most striking difference is the appearance of a distinct metachromasy band at 506 nm in the bound CV. If such a band were also involved in the spectra of the bound TF, it would be very weak in intensity and exist in the wavelength region shorter than the 374—370 nm isosbestic point. (No apparent metachromasy band appears for either of these two dyes in the UV region). Difference is also noted for the variation of the peak positions in the presence of NaPP. The 451 nm band of TF steadily shifts hypsochromically as indicated by $\Delta\lambda$ (Figs. 1—3), whereas in the bound CV the original band at 592 nm, a shoulder at about 550 nm, and the new metachromasy band at 506 nm remain unshifted.[1,2]

Except for the differences described above, both TF and CV show the following common features. A long wavelength isosbestic point is noted at 479—475 nm for TF and at 637—639 nm for CV. The existence of such a point should imply that there is an absorption band in the wavelength region greater than that of the major band. A new long wavelength band has been observed in some triphenylmethane and acridine dyes in the solid state.[19] The P/D dependence of the metachromatic change of the two systems is also alike (Figs. 1 and 3 in Ref. 2; Figs. 1, 3a, and 3b in this article); for example, at very high and low P/D values, the absorbance of either system approaches that of the corresponding dye alone regardless of the polymer chain length. Another similarity is noted for the chain length dependence of the metachromatic change. For either dye the critical chain length of NaPP is rather short in terms of the number-average degree of polymerization. If the midpoint of the ε *vs.* $\bar{n}$ curves (Fig. 4) is taken to be a measure of the chain length dependence of metachromasy, the critical chain length is slightly shorter for TF ($\bar{n}=10$) than for CV ($\bar{n}=14$—16).[2]

For the NaPP sample whose $\bar{n}$ value is either 154, 24, or 11, a given P/D value of, say 310, signifies a polymer–dye solution containing on an average about two, thirteen, or twenty-eight polymer molecules per dye, respectively. The observed metachromatic change of TF in the solution is not abrupt but only gradual in the P/D range 100—1000 (Fig. 3). The result is best interpreted by assuming that the binding of TF to the polymer site is statistically random and essentially independent of the kind of site. If a preferential binding occurs to the dissociated secondary phosphate at the chain terminal of NaPP (only one terminal is fully dissociated at neutral pH),[20] the ε *vs.* P/D curve would show an abrupt change at a P/D value which nearly equals the $\bar{n}$ value of a given NaPP sample. Apparently this is not the case (Figs. 3a—3b). Thus, the present data on TF is not in accord with the notion that acridine orange is bound preferentially by the terminal doubly charged phosphates in the polynucleotide–acridine orange complexes.[21,22]

As observed for CV,[2] the binding of TF to NaPP is of electrostatic nature.[1,23] This is indicated from the results of the effect of small ions on metachromatic behavior (Figs. 5a and 5b) and conductometric titration (Figs. 6a—6c). It is clear from a comparison of Figs. 5a—5b with the result of the previous study on CV[2] that TF requires slightly more Na^+ ions than CV to dissociate from NaPP. The chemical structure of TF may be responsible for this result. Once bound to the polymer site, the quaternary ring nitrogen (the most probable binding locale) of TF may be shielded effectively from the small ions by two amino groups at the 3- and 6-positions, while the positively charged dimethylamino nitrogen of CV (a quaternary[24] or quinonoidal iminium ion[25]) could be exposed more to solvent and consequently susceptible to Na^+ ions.

Comparison of Metachromatic Behavior between Certain Acridine Dyes in the Presence of NaPP. Interaction with NaPP has been studied in some detail for acridine orange,[9—12] proflavine[9] and TF. The metachromatic behavior of the NaPP–proflavine system is very close to that of the NaPP–TF system. This is understandable considering the chemical structure of proflavine (protonated rather than methylated at the ring nitrogen but otherwise identical with TF). The spectral change of the visible band of the NaPP–acridine orange system differs from that of the NaPP–TF system; a broad, new band appears at 450 nm at the expense of the intensity of the principal 492 nm band as P/D values decrease.[10] This is somewhat analogous to the metachromatic behavior of the CV bound to NaPP.[2] Acridine orange possesses two dimethylamino groups, and proflavine or TF has two amino groups at the 3- and 6-positions, the pK values being 10.45, 9.65, and >12, respectively.[26] Thus, the difference and similarity of the observed metachromatic behavior should be sought from these two factors.

In spite of a qualitative variety of the spectral characteristics, the quantitative aspects of the metachromatic behavior of these acridine dyes are remarkable alike. The ε *vs.* P/D curves of acridine orange bound to NaPP indicate that the metachromatic change is maximum in the P/D range 2–20 (The Kurrol salt)[10] and 1—20 ($\bar{n}=110$),[11] and approximately 20—40 ($\bar{n}=9$)[10] and 10—40 ($\bar{n}=12$)[12] for unfractionated oligomeric samples. No metachromatic change was observed for the NaPP sample with $\bar{n}$ less than 5[10] or 3.[12] As P/D values increase to 10^4, the molar extinction coefficients of the bound dye approach the value of free acridine orange.[10] Since all the results compare favorably with the present data of the NaPP–TF system and the present work has established the fact that TF is totally bound to NaPP, acridine orange should also remain bound to NaPP even at exceedingly high P/D. The stoichiometry of the binding of the flat, symmetrical acridine dyes with substituents at the 3- and 6-positions appears to be general for NaPP.

Closing Remarks. Together with the results with malachite green[1] and CV,[2] the present study of the interaction between NaPP and TF reveals the following. The metachromatic behavior depends on the chain length of NaPP, and the critical range of the degree of polymerization lies from 7 to 20. The dye is definitely bound to the polymer site at extremely high P/D values, irrespective of the appearance of the absorption spectra which do not appreciably differ from the free dye spectra. Although a closely resembling situation results from the addition of a great deal of the small ion to the NaPP-dye solution, the bound dye is compelled to dissociate from the polymer site because of the competition with the small ion. Thus, the resulting spectra are nearly the same in these two cases, but the underlying processes are proved to be different. It is thus concluded that the binding mode of each dye to NaPP is general and arises from complicated electrostatic interactions for which the positively charged nitrogen moiety of the dye is mostly responsible. The spectral behavior is remarkably characteristic of individual dyes, even though they belong to the same structural class.

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