Metachromasy. II.* Interaction between Sodium Polyphosphates and Crystal Violet with Special Emphasis on the Effect of Chain Lengths of the Polymer on the Metachromasy Band

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The interaction of crystal violet with sodium polyphosphate in aqueous solutions at neutral pH has been investigated by means of visible absorption spectroscopy and electric conductivity. The mixing ratio of polymer residue to dye was varied from 0.1 to 10000. The metachromasy band at 506 nm was found to fully develop between 3 and 10, whereas conductometric titration showed that crystal violet saturates the polymer site. Emphasis was placed on the effect of the polymer chain length on the metachromasy band. To study this effect, 17 fractionated and 13 refractionated polymer samples with degrees of polymerization extending from 4 to 300 were utilized. A critical range of the degree of polymerization from the metachromasy band exists between 7 and 20. The effect of small ions on metachromasy was also studied using sodium phosphates. An increase in Na^+ ions tends to suppress the metachromasy band. This effect is more pronounced with shorter polymer lengths. In order to explain the observed metachromasy phenomena, two mechanisms were postulated; one attributes metachromasy to the flexibility of the polymer backbone when dye is present, and the other deals with specific interaction of dye and polymer site.

A characteristic change of color is exhibited by certain cationic dyes in aqueous solutions, as their concentration is increased or the temperature is lowered. A similar change occurs when such dyes are bound to certain polyelectrolytes with anionic groups in low concentrations where the dyes alone obey Beer's law. This is the phenomenon of *metachromasy*, as originally termed by Ehrlich, in contrast with *orthochromasy* which signifies the relative lack in such a color change of basic dyes at higher concentrations or when bound to polyelectrolytes.[2] The metachromatic change, which is most pronounced in the visible spectrum, is frequently characterized by the appearance of a new absorption band (or bands)—metachromasy band—or prominent shoulders in the wavelength region shorter than the original band of a pure dye. The change has been interpreted as due to the formation of various aggregates of the bound dye molecules on the polymer sites.[3]

Such metachromatic changes are frequently specific rather than general and constitute an experimental basis of histo- and cytochemical applications. For a particular dye the metachromatic behavior is characteristic of the polyelectrolyte species and its conformation under given experimental conditions.[3-5] For a particular polymer species it often depends on the specific substituents attached to the dyes even if they belong to a class of the same chemical structure.[6,7] The differentiation of nucleic acids between themselves or from proteins *in vitro* as well as *in vivo* is based on the delicate color shades of a bound metachromatic dye.

A simple polymeric model of nucleic acids is indisputably polyphosphoric acid which is one of the simplest polyelectrolytes with ionizable phosphate groups in the polymer chain. The interactions between polyphosphates and metachromatic cationic dyes have been studied and the results compared with the interaction with nucleic acids. Dyes studied so far are toluidine blue,[8] 4,5,4′,5′-dibenzo-3,3′-diethyl-9-methylthiacarbocyanine,[9] acridine orange,[3,10-14] and proflavine.[10] Polyphosphates utilized in these studies were randomly chosen from small oligomeric samples to very high polymeric Kurrol's salts. Few attempts, however, have been made to find the relation between the critical polymer dimension and metachromasy.

In this paper we present, for the first time, detailed results of the effect of the length of polymer chain on induction of metachromasy and, in particular, evidence for the existence of a critical range of the chain lengths. We prepared a large number of fractionated polyphosphate samples with degrees of polymerization in the range 4—300. As a metachromatic dye we chose crystal violet, purer and more representative of triphenylmethane dyes, instead of acridines. The optical properties of crystal violet have been studied to the same extent as acridine orange or proflavine both in the absence [15-17] and presence of various polyelectrolytes.[6,7,18-20,25,26] Crystal violet is particularly suitable, since, under certain conditions, it exhibits a new, well-defined metachromasy band at 506 nm separated from its principal band, when bound to polyphosphate.[1]

Experimental

Materials. Sodium polyphosphate (NaPP) was synthesized from sodium dihydrogen phosphate used as a monomer by the method of pyropolymerization.[1] The degree of polymerization of each preparation was controlled by adding an appropriate amount of disodium hydrogen phosphate, as a chain terminator, to the monomer. The reactants were mixed and preheated in a platinum crucible below 200 °C to dryness and then heated at 800 °C in an electric furnance for about 8 hr. The melt was then quickly solidified in a liquid nitrogen bath.

Eight NaPP preparations were each fractionated into five to eight fractions of approximately equal volume by the method of fractional precipitation from aqueous solution (ca. 7%) with acetone. Each fraction was separated from the mother liquor by decantation, redissolved in water, lyophilyzed, and dried *in vacuo* at room temperature and then at 57 °C. Samples were stored in a desiccator over

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phosphorus pentoxide. The number-average degree of polymerization, $\bar{n}$, of the total fifty fractions was determined by the method of end-group titration with sodium hydroxide and found to be in the range 4—300. In order to prepare better-defined samples with respect to molecular weight distribution, a mixture of four fractions, whose $\bar{n}$'s were in the neighborhood of 11, was refractionated into eight fractions. Another mixture of two fractions with $\bar{n}$'s of approximately 120 was also refractionated into eight fractions.

Crystal violet (CV) in the chloride form (Chroma Gesellschaft, Schmid & Co.) was purified by recrystallization from hot water and dried to be anhydrous at 80 °C *in vacuo*.[1] The purity of CV was tested by thin-layer chromatography (1: 4 methanol–chloroform mixed solvent as an eluent). The vacuum-dried dye sample was weighed as anhydrous chloride to prepare a stock solution, which was stored in the dark at room temperature. All glassware was equilibrated with a concentrated dye solution and rinsed thoroughly with water before use to avoid the undesirable adsorption of dye. The maximum molar extinction coefficient at 592 nm was 94000 at 1.12×10^{-5}M.

Procedures. An anhydrous polymer sample was dissolved in distilled water to prepare a stock solution which was stored in the cold. Just prior to measurements, one part of the dye stock solution was added dropwise to one part of the polymer solution, while the mixture was being stirred. The dye–polymer solution was then equilibrated for about 15 min. This order of mixing was observed throughout. The final concentration of dye in the dye–polymer solution was maintained at a fixed value of 1.12×10^{-5}M, unless otherwise stated. The ratio of monomer residue (or anionic site) to dye, P/D, was defined as before.[4]

The buffered dye–polymer solution was prepared by using the Na_2HPO_4–NaH_2PO_4 buffer system, the components of which are both monomeric units of NaPP. One part of the buffer solution was first added to one part of the polymer solution, followed by two parts of the dye solution. The pH of buffered dye–polymer solutions was adjusted to about 6.5. Part of this solution was saved for pH determination. The final buffer concentration was kept at five times higher than that of NaPP, except for the study of the effect of concentration of added salt (Na_2HPO_4–NaH_2PO_4) on the dye–polymer complex formation.

Measurements. A Hitachi Model EPS-3T recording spectrophotometer was used together with a matched pair of 1 cm long quartz cells. The temperature was controlled at 25 °C by circulating regulated water through a cell holder. The solution was always equilibrated for 5—10 min before the measurement of an absorption spectrum which was scanned at least twice to ascertain reproducibility. Conductometric titration was carried out on a Jones type 1000-Hz bridge.[21,22] The electrodes of a conductivity cell with a cell constant of 0.0333 were made of bright platinum to avoid the adsorption of NaPP. No time dependence of the cell resistance was observed in the presence of the solution of NaPP or CV. 1 ml of a 1.3×10^{-3}M NaPP solution was added (0.04 ml at a time) through a burette to 25 ml of a 2.26×10^{-5}M CV solution in the cell. The mixture was stirred and allowed for about 2 min each time for equilibration before reading the resistance.

Results

Spectra of Crystal Violet in the Presence of NaPP in Aqueous Solutions. The visible absorption spectra of CV in the presence of a high molecular weight NaPP are shown in Fig. 1. The values of P/D were varied by adjusting the polymer concentration at a fixed CV concentration. The absorption maximum of CV at 592 nm decreases with an increase in P/D values up to 3 by the addition of NaPP, while its absorption at 506 nm increases steadily to a distinct band—a metachromasy band. There is an isosbestic point at 639 nm in the longer wavelength side in a family of the NaPP–CV spectra at P/D values less than unity. The spectral trend reverses very slowly at first and then gradually, as the polymer concentration is increased to about 100 in terms of P/D. At exceedingly high P/D values, the 592 nm band regains its intensity, whereas the metachromasy band at 506 nm diminishes. Thus, the overall spectrum becomes almost the same as that of pure CV. An isosbestic point also appears at 636 nm in the spectra of NaPP–CV systems at P/D values higher than 100. It should be noted that in the spec-

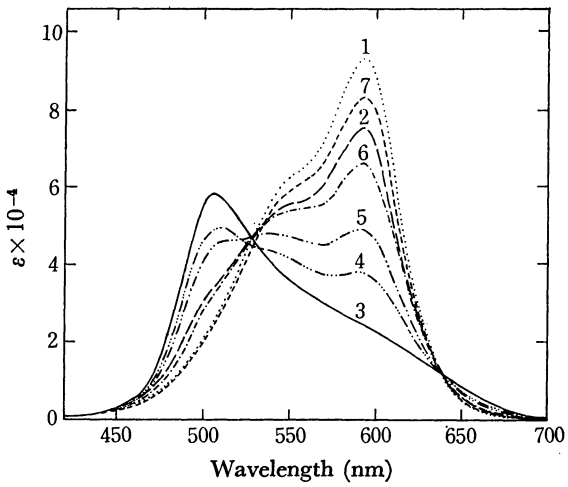


Fig. 1. Spectra of CV in the presence of NaPP ($\bar{n}$=154) at various P/D values in aqueous solutions.
(1) Pure CV (P/D=0) for comparison, (2) P/D=0.3, (3) P/D=3, (4) P/D=80, (5) P/D=150, (6) P/D=300, (7) P/D=1000.

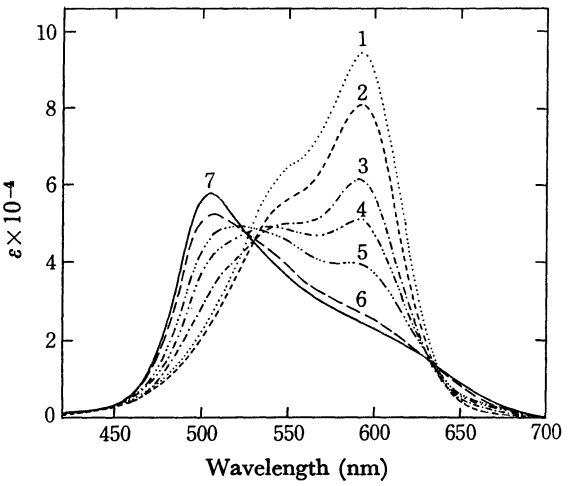


Fig. 2. Spectra of CV in the presence of NaPP with various chain lengths at a P/D value of 3 in aqueous solutions.
(1) Pure CV (P/D=0) for comparison, (2) $\bar{n}$=7.77, (3) $\bar{n}$=12.9, (4) $\bar{n}$=15.6, (5) $\bar{n}$=24.4, (6) $\bar{n}$=63.0, (7) $\bar{n}$=154.

trum of CV there is a weak, probably forbidden band between 340 and 380 nm which is also affected by the presence of NaPP.[1] No quantitative analysis of this band is given because of the small changes in intensity and band position.

The effect of the chain length of NaPP on the visible absorption spectra of CV is shown in Fig. 2. The P/D value is held at 3 which is one of the most favorable values for the appearance of the metachromasy band. The spectra for $\bar{n}$ values less than about 5 are essentially the same as the pure CV spectrum. As the values of $\bar{n}$ increase, the principal band intensity at 592 nm decreases, while the metachromasy band intensity increases for the NaPP samples. A sharp transient region of $\bar{n}$ lies approximately between 7 and 20. The intensities of both bands become unchanged as $\bar{n}$ exceeds 20. It is interesting to note that the spectral behavior shown in Fig. 2 is closely related to that shown in Fig. 1. This indicates that, with respect to the 506 nm metachromasy band, some correlation exists between changes in P/D values at a given $\bar{n}$ and changes in $\bar{n}$ at a given P/D value. All results are hereafter described in terms of absorptions at the 592 and 506 nm bands with which the metachromasy of CV can be characterized most conveniently.

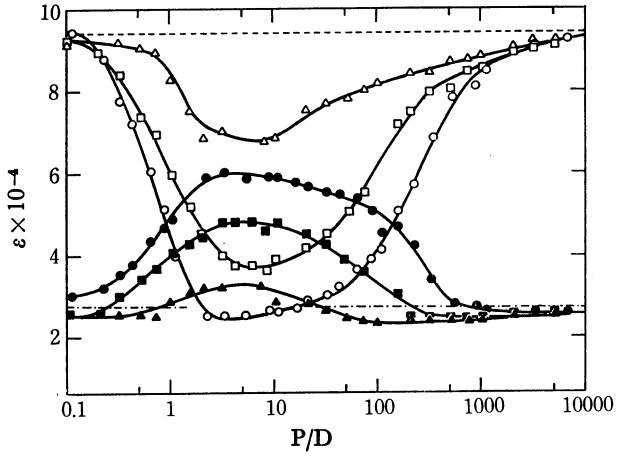


Fig. 3. The dependence of metachromasy on the P/D values for NaPP–CV systems with different chain lengths in aqueous solutions.
Open symbols are the molar extinction coefficients of CV at 592 nm and filled ones are those at 506 nm. -○- and -●- for $\bar{n}$=154, -□- and -■- for $\bar{n}$=24, -△- and -▲- for $\bar{n}$=11.

Effect of the P/D Values on the Absorption of NaPP–CV Complexes with Various Degrees of Polymerization. In Fig. 3, the molar extinction coefficients of CV at 592 and 506 nm in the presence of three different NaPP samples ($\bar{n}$=154, 24, and 11) are plotted against P/D on a logarithmic scale. The effect of P/D is such that, at both low and extremely high P/D values, the molar extinction coefficients at 592 and 506 nm are about the same as those of pure CV. The 506 nm band becomes most outstanding and the 592 nm band is diminished to a minimum for each NaPP sample at P/D values between about 3 and 10. The extreme values of the extinction coefficients differ with the chain length of NaPP. This indicates that, while the metachromasy is developed to be maximum for each NaPP sample in this P/D range, the magnitude of the 506 nm metachromasy band is affected by the polymer chain length. The three NaPP samples were so chosen that the metachromasy band is induced to a small ($\bar{n}$=11), medium ($\bar{n}$=24), or large ($\bar{n}$=154) extent.

Effects of the Chain Length and Fractionation of NaPP on the Absorption of NaPP–CV Complexes at Various Values of P/D. The effects of chain length and frac-

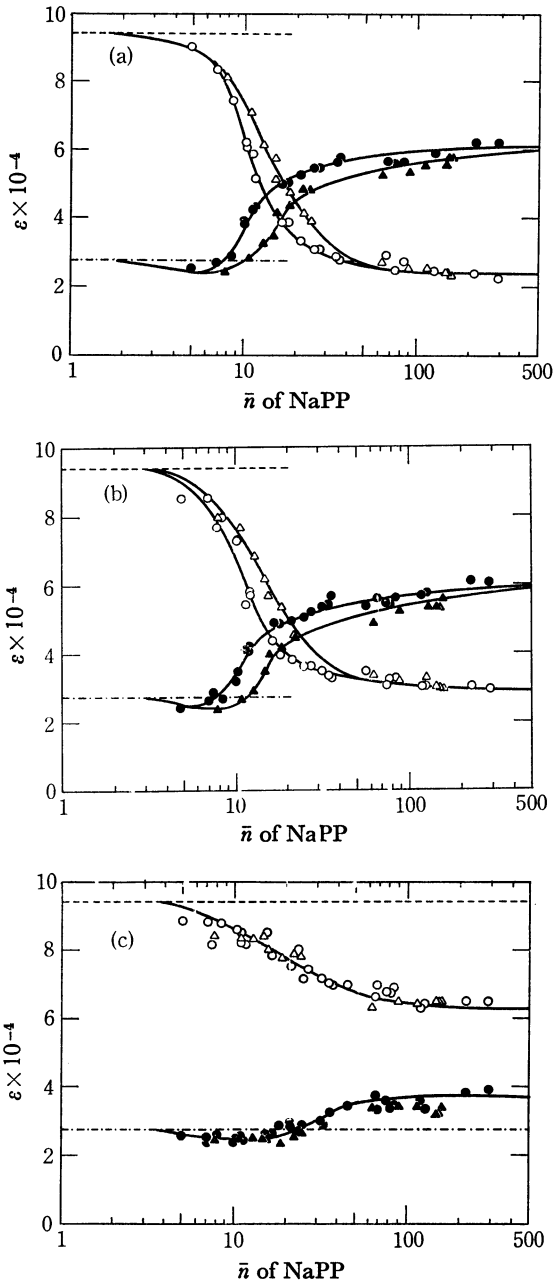


Fig. 4. The dependence of metachromasy on the chain lengths of fractionated and refractionated NaPP samples at (a) P/D=3, (b) P/D=30, and (c) P/D=300 in aqueous solutions.
In each figure, open symbols are the molar extinction coefficients of CV at 592 nm and filled ones are those at 506 nm. -○- and -●- for fractionated NaPP, and -△- and -▲- for refractionated NaPP.

tionation on the induction of metachromasy band are shown in Figs. 4a—4c, where the molar extinction coefficients of the NaPP–CV system are plotted against the chain length of both fractionated and refractionated NaPP samples on a logarithmic scale. The concentration of each NaPP sample is so chosen that the metachromasy is most distinctive (P/D=3), medium (P/D=30), or very weak (P/D=300). At P/D values of 3 and 30 the principal band at 592 nm decreases, while the metachromasy band at 506 nm increases, very sharply in a narrow range of the chain length 7—20. Both band intensities then level off, as $\bar{n}$ reaches about 100. The midpoints of these sigmoidal changes in ε_{506nm} and ε_{592nm} correspond to $\bar{n}$=17 and 14 (P/D=3), and $\bar{n}$=18 and 16 (P/D=30), respectively, for refractionated samples. The midpoint is slightly displaced to $\bar{n}$=30 and 20 at a P/D value of 300, which indicates that a longer chain length is required for metachromasy. In any case, it is evident that metachromasy is dependent on the polymer chain length and is critically associated with the oligomeric dimensions.

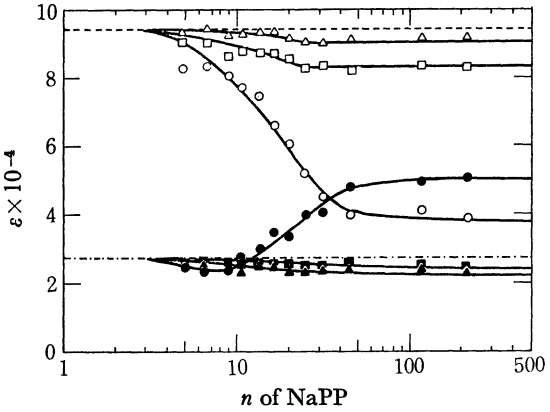


Fig. 5. The dependence of metachromasy on the chain lengths of NaPP at various P/D values and at fixed pH's.
Open symbols are the molar extinction coefficients of CV at 592 nm and filled ones are those at 506 nm. -○- and -●- for P/D=5 at pH=7.0, -□- and -■- for P/D=50 at pH=6.8, -△- and -▲- for P/D=500 at pH=6.5.

The pH value of NaPP in aqueous solutions varies slightly with the chain length at a given value of P/D and the polymer concentration at a given value of $\bar{n}$. An attempt was made to examine the effect of $\bar{n}$ on metachromasy at a constant pH using a sodium phosphate buffer five times more concentrated than NaPP (Fig. 5). As the data in Figs. 4a—4c and 5 indicate, the optical behavior is all the same except that the development of the metachromasy is somewhat suppressed in the buffered solution. This is, not a direct consequence of maintaining pH at a fixed value*,

* The slight variation of pH in the neutral region is due to the fact that the degree of ionization of end-groups of a polymer chain (other phosphate residues are all ionized in this pH region) depends on the chain length. For a given NaPP sample, the metachromasy is confirmed to be independent of pH between 5.5 and 9 for CV.

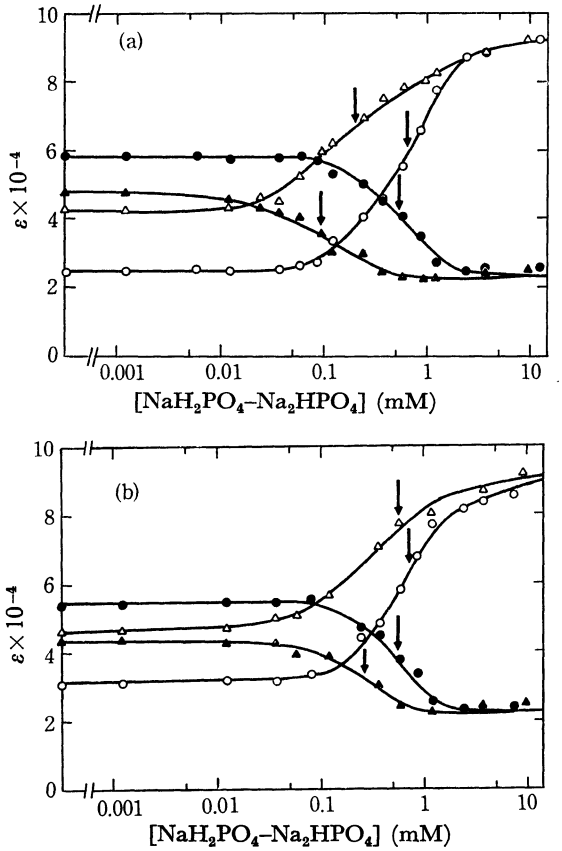
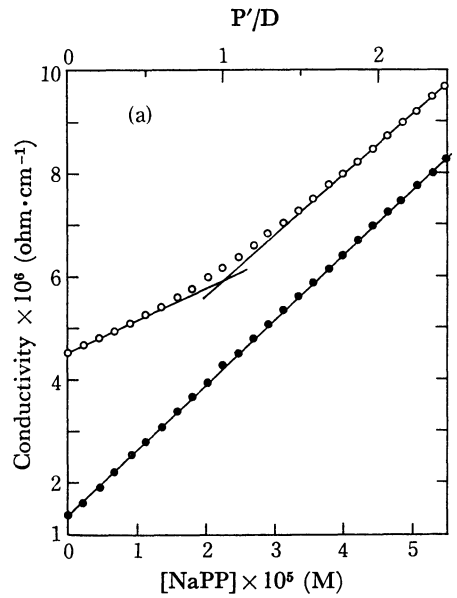


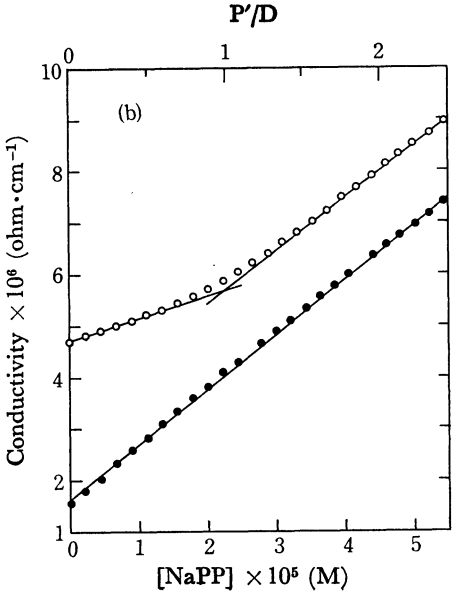
Fig. 6. The effect of small electrolytes on metachromasy at (a) P/D=3, and (b) P/D=30.
Open symbols in each figure are the molar extinction coefficients of CV at 592 nm and filled ones are those at 506 nm. -○- and -●- for NaPP with n=154, and -△- and -▲- for NaPP with n=24. Arrows indicate the mid-points of the changes in ε.

but due to the effect of the ionic strength of the buffering small ions on metachromasy (*vide infra*).

Effect of Addition of Small Electrolytes on Metachromasy. The effect of small ions on the absorption bands at 592 and 506 nm is shown for two NaPP samples ($\bar{n}$=154 and 24) at P/D values 3 and 30, respectively (Figs. 6a and 6b). At each P/D the effect of the concentration of the added small ion on metachromasy is insignificant up to about 1×10^{-5} M, a concentration comparable with that of CV. However, a further increase in the salt concentration gives rise to a gradual decrease in metachromasy. The absorptions at 592 and 506 nm become indistinguishable with those of pure CV at salt concentration higher than 10^{-2} M, up to which the pure CV spectrum is independent of the amount of added salts. The chain length effect on this process, which has been attributed to the competitive binding between CV^+ cations and Na^+ ions toward polymer sites,[1,19] reflects the prevailing relationship between molecular weight of polymer and metachromasy. The 506 and 592 nm sigmoidal curves for the higher $\bar{n}$ sample are clearly displaced to the right. More small ions are required to dissociate the bound dye. The arrows in Figs. 6a and 6b indicate the midpoints of either increasing or decreasing curves of molar extinction coefficients.

Conductometric Titration of CV with NaPP of Three Different Chain Lengths. In order to establish the relation between the binding of CV to polymer site and the chain length of NaPP, conductometric titrations of CV with representative NaPP samples were carried out. The results are shown in Figs. 7a—7c, where each corresponding blank titration is also plotted. In the absence of CV the conductivity of each NaPP sample increases with the increase of the concentration, although the slope depends on the chain length. In the presence of CV, however, the conductivity of the titrated solution increases in two steps on the dropwise addition of the NaPP titrant. A sharp break always appears at a P/D value of about unity (1.01, 1.01, and 1.02) for each NaPP sample ($\bar{n}$=11, 24, and 154) as shown by an intersection of two interpolated lines (Figs. 7a—7c). This indicates that, regardless of the chain length of NaPP, *i.e.*, either oligomeric or polymeric sample, CV binds to the NaPP site at most up to a one-to-one ratio at a total CV concentration of





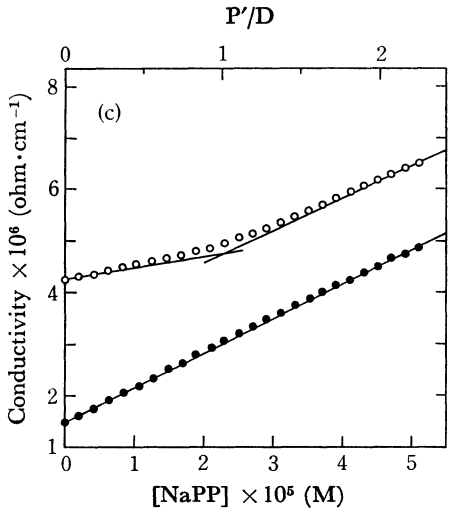


Fig. 7. Conductometric titration of CV with NaPP with various chain lengths (a) n=11, (b) n=24, and (c) n=154.
-●- for blank solution titrated with NaPP for comparison. The concentration of NaPP is corrected for volume increase. P' is the concentration of the ionized group of the polymer and corrected as equal to $P(1+1/n)$ where P is the residue concentration. Other conditions are given in Experimental.

2.26×10^{-5} M. After the P/D=1 break, a further addition of NaPP increases the conductivity of the NaPP–CV solution practically in parallel with the increase in conductivity of a pure NaPP solution.

Discussion

Effects of Mixing Ratio and Polymer Chain Length on Metachromasy. The fact that an abrupt change in metachromasy of CV occurs in the presence of NaPP with chain lengths in the range 7—20 at P/D values of 3 and 30 (Figs. 4a—4b) should be taken as evidence that a certain degree of polymerization is required for a polyphosphate to show metachromasy in a dilute concentration range of 10^{-5} M. This finding newly confirms the previous incomplete reports[8,11,12] and definitely establishes the existence of a critical range of polymer chain lengths for metachromasy. It is not known at present why the polyelectrolyte should have a certain degree of polymerization to induce metachromasy, or why such a critical range should lie in the relatively short oligomeric dimensions. However, there can be no doubt that some action of water on the NaPP–CV complex is involved. The conformation of the complex in aqueous media can be strongly affected by factors such as dielectric constant, hydration and dissociation. Such effects may vary with the chain length of polymer—less pronounced in the case of higher molecular weight. Gekko reported a sudden change in the second virial coefficients of dextran oligomers in aqueous solutions.[23] He attributes the sharp break, which occurs at a degree of polymerization close to 12, to the conformational transition from uncoiling stretched-form for lower molecular weight to random-coil form for higher molecular weight

oligodextrans. Goodman *et al.* have also demonstrated that a critical range exists between $\bar{n}=7$ and 9 for γ-methyl-L-glutamate oligomers forming a helix in dimethylformamide.[24]

The effect of the refractionation of NaPP samples is apparent from Figs. 4a—4c. From the data which show that the sigmoidal curves of the samples are shifted toward the higher $\bar{n}$ relative to the fractionated ones, each refractionated sample should have a narrower molecular weight distribution. This is because the ability of inducing the metachromasy band is unproportionally high with the longer chain length components, which conceivably are included in larger proportions in once-fractionated oligomeric samples than in the twice-fractionated ones. We have studied competitive interactions between a higher ($\bar{n}=154$) and a lower ($\bar{n}=11$) molecular weight NaPP toward the binding of CV. The presence of the small amount (10%) of the higher NaPP in the mixture is sufficient to produce spectra identical with those of the higher NaPP–CV complex at several P/D values. The molecular weight distributions have thus been estimated to be reasonably narrow enough for establishing the critical range of the chain lengths.

Comparison of Optical Titrations with Conductometric Titrations. The optical titration curves in Fig. 3 can be divided into two parts; the region of P/D smaller than unity, where CV in solution is in excess of NaPP residues, and the rest of P/D, where the situation reverses. This option can be justified particularly for polyphosphates, since the only functional group is ionizable phosphates. The first region represents, at a fixed dye concentration (1.12×10^{-5} M), the state of equilibrium between free and bound dyes in solution. This is manifest when the pure CV spectrum is compared with any one of NaPP–CV spectra at very low P/D. They are essentially superimposable. The 592 and 506 nm band intensities are restored almost linearly with P/D between approximately unity and zero. An isosbestic point exists at 639 nm. Hence, it is unlikely that any strong interaction is involved between unbound and bound CV molecules.

With regard to the transition from the first to the second region, the present results (Fig. 3) corroborate the fact that not only the magnitude of metachromasy but also the break at a P/D value near unity depends on the chain lengths of oligomeric polyphosphates ($P/D=1.3$, 1.6, and 2.0 with the uncertainty of about ±20% for $\bar{n}=154$, 24, and 11 in this order). The conductometric data (Figs. 6a—6c) show a sharp break at a P/D value of about unity for each NaPP sample. The break is almost independent of the chain length (P/D=1.01, 1.01, and 1.02 with the uncertainty of about ±5% for $\bar{n}=11$, 24, and 154 in this order). The unexpected conductometric finding may be taken to be an indication that CV can bind to the phosphate residue of the polymer up to the 1:1 ratio at maximum, *i.e.*, the saturation of sites irrespective of the polymer chain length; thus a simple electrostatic binding between cationic CV moiety and anionic phosphate residue should not be the direct cause for the generation of metachromasy. If such an electric charge neutralization by the Coulombic force were sufficient to give rise to metachromasy, then the fact that the metachromasy is less developed for smaller NaPP samples could not be correctly explained without modifying the interpretation of the conductometric data.

Critical Views of the Origin of Metachromasy. As regards the restoration of the 592 nm band intensity observed at higher P/D portion of the second region where polymer sites are in large excess of dye, Bradley *et al.* proposed a mechanism in which metachromasy is generated as a result of the interaction between adjacent bound dyes on the sites of a polymer of infinite length.[3] As the number of the polymer sites becomes in great excess of dye molecules, the bound dyes are unstacked from the adjacent sites and tend to redistribute themselves on the sites and, consequently, they are separated from one another by some vacant sites on a single polymer. Or they are transferred to the isolated sites of other polymer molecules. Pal and Chaudhuri have considered the interaction between adjacent bound dyes to be of hydrophobic nature.[25] According to the original stacking theory, however, the magnitude of metachromasy induced by CV bound to NaPP should remain practically constant over a wide P/D range (particularly between 1 and 10) irrespective of the polymer chain length. This conclusion apparently contradicts the present experimental results.

We alternatively offer two possible but tentative views on the origin of metachromasy, recognizing in both the important role of solvent water molecules. The first view is a modified version of the stacking theory which associates metachromasy with the conformation, more specifically flexibility, of the polymer backbone to which dyes are bound. Since the high molecular weight NaPP is more flexible, the sparsely bound dyes may approach one another while being shielded from hydrophilic environments even in a relatively high P/D range. The forces are thus exerted between the approaching dyes sufficiently to induce metachromasy. Low molecular weight oligomers are too short to be flexible. Because of the stiffness of the short chain, the bound dyes can hardly be enclosed so that they are adjacent. When all the polymer sites are filled with dye molecules (P/D=1), the chain of either short or long polymer would have to be elongated stiffly rather than compactly coiled because of the geometric constraint of the dye-bound moiety. Therefore, the development of metachromasy is optimum in the P/D range 3—10 rather than near unity. One drawback is the difficulty of explaining the fact that the metachromasy band position at 506 nm remains independent of P/D values over almost the entire range while only its intensity varies, and that CV shows a metachromasy band with NaPP, but malachite green does not.[1]

The second view is the dye-site interaction theory which emphasizes the local and specific binding resulting from the dye-site interaction more than the general effect of gross polymer conformations and bound dye-bound dye interactions. The theory attaches primary importance to the following factors: the specific chemical

structure and symmetry of a dye, alteration of the electronic states of dye and site upon formation of a bond which is more than a pure Coulombic one, and the distortion of the bond by some steric perturbation which should increase as more dye molecules are bound in a given crowded area (the lower P/D region). When the bonding between dye and site is purely electrostatic with little orbital overlapping and unsheathing of hydrated water, no metachromasy band should be expected (higher P/D region). In this case, the overall electronic structures of the excited and ground states of the bonding site probably closely resemble those of the pure CV in organic solvents. The second theory does not exclude contributions, to metachromasy, of the flexibility of the polymer chain, which should increase the steric constraints on the bound dye as the chain length increases (high $\bar{n}$ samples), and of the bound dye-bound dye interaction which is probably affected by the conjugated π-electron system of triphenylmethane skeleton. These factors are not to be sufficient for elucidating metachromasy; thus, the first view may be considered to be supplementary to the second.

Closing Remarks. The primary importance should be the class and functional substituent of triphenylmethane dyes for metachromatic behavior. While malachite green with a C_{2v} symmetry, which is short of a substituted amino group as compared with CV, behaves very similarly in the presence of NaPP but shows no metachromasy band under the closely related conditions, ethyl violet and parafuchsin, both of which are homologs of CV with a point symmetry of D_{3h}, show the metachromasy band. In this connection, systematic work relating the development of metachromasy to the chemical structures of triphenylmethane and acridine dyes is highly desirable.[6]

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