A. **Mercuric chloride as chromotrope**:

Holmes (1926) first noticed that the basic dyes which do not conform to Beer's law are capable of producing metachromasia in appropriate tissues, and that this metachromatic color is similar to the color of the more concentrated dye solutions. Aqueous solutions of the potentially metachromatic dyes show changes in the shapes of their absorption spectra as the dye concentration is raised from $10^{-5}$ toward $10^{-2}$M (Sheppard and Geddes (1944), Rabinowitch and Epstein (1941)). At dilute end of this range they usually have a single high peak (called the orthochromatic or the $\lambda$-band), at higher concentration the $\alpha$-band becomes depressed and a new band ($\beta$-band) appears at shorter wavelength and at the highest concentration that can be reached in aqueous solution both $\alpha$-band and $\beta$-band are usually depressed and a third band ($\gamma$-band or $\mu$-band) is emerged at still shorter wavelength (Michaelis (1950), Schubert and Levine (1955)) corresponding to the higher aggregates of the dyes. A chromotrope has the ability to induce metachromatic spectral change in an orthochromatic dilute aqueous solution of the basic dye through the formation of a metachromatic compound. The metachromatic compounds exist in solution in equilibrium with their component cations and polyanions, and have absorption peak ($\mu$-band) at a wavelength 50-100 nm less
than the wavelength of the peak of the dye alone in dilute aqueous solution ( < \text{K-band} \rangle (\text{Fig. 1}). The common chromotropes are polyanionic in nature and the metachromatic color change induced in the dye solution is through the formation of a compound between a chromotrope polyanion and dye cations having 1:1 stoichiometry. The 1:1 stoichiometry in the compounds of various chromotropes and cationic dyes has been established by various methods:

A. Conductance measurement (Schubert and Levine (1953)).
B. Equilibrium dialysis (Levine and Schubert (1952)).
C. Spectrophotometric titration (Bradley and Wolf (1959), Stork et al (1973), Vitagliano et al (1973)).
D. Removal of the metachromatic compound from aqueous solution by immiscible organic solvents (McIntosh (1941), Pal and Schubert (1961b)).
E. Separation of the metachromatic compounds by ultracentrifugation (Pal and Schubert (1961a)).
F. Specific adsorption of the metachromatic compounds by inorganic salts (Pal and Schubert (1961b), (1962)).
G. Pulse radiolysis techniques (Moore et al (1970)).

All these methods point to the formation of 1:1 compound between the dye cations and a polyanion molecule to exhibit metachromasia and presumably this interaction is electrostatic in nature. The methods D, E, F also point to the surprising fact that even in the presence of moderate excess of a polyanionic
chromotrope, the dye cations prefer to occupy sites with adjacent neighbours indicating the strong aggregating tendency of a metachromatic dye. When dye cations occupy adjacent sites on a chromotrope molecule they suffer aggregation associated with intermolecular bonds between the adjacent dye cations which may be extended over the entire chromotrope molecule if the bound dye cations are stacked extensively. The minimum number of the dye ions to be aggregated to exhibit metachromasia is not established, but this minimum number is not two since dimerisation gives rise to the intermediate \( \beta \) - band only (Armstrong, Kurucsev and Strauss (1970), Mukherjee and Ghosh (1970)). Since smaller polyanions like inositol hexasulfate, - hexaphosphate, ammonium molybdate etc. may act as chromotropes (Pal and Biswas (1971)), Mandal, Biswas and Pal (1969), a chromotrope need not be a macromolecule. Miura and Kubota (1967) report that a degree of polymerisation greater than nine is adequate to induce a chromotropic ability in polyphosphate. Binding of dye cations at the anionic sites of a chromotrope is an essential though not the only condition of exhibiting metachromasia. Dyes like methyl green, methylene green, capri blue are bound to the polyanions, but without exhibiting spectral changes. It seems that the bound dye cations must also be stacked properly to exhibit metachromasia and the relation between the stacking tendency of a dye and its structure or rather the relation between the structure and
metachromasia has not been established. So any theory that proposes to interpret the metachromatic spectral shift, only deals with the spectral shifts of the potentially metachromatic dyes. The accepted theory is that the dye cations are bound to the polyanion at the adjacent sites and are stacked through dye-dye interactions which may be hydrophobic in nature (Pal and Schubert (1963), Mukherjee and Ghosh (1963), Mukherjee and Ray (1963), Rohatgi and Singhal (1966)). The local regularity of the water molecules forming so called "iceberg" or "flickering clusters" model of water structure as proposed by Frank and Evans (1945) and Frank and Wen (1957) plays an important role in this entropy effect. Mukherjee and Ghosh (1970) suggest that dimerisation of methylene blue in aqueous solution is associated with an increase of entropy of 3.5 entropy units by breaking some of the icebergs around the monomer. The dimerisation of the dye would therefore be due to hydrophobic interaction and the force driving the dimerisation would be entropic in nature. Dimerisation of certain dyes like diidofluorescein, eosine, erythroxine and rose bengal also involves the hydrophobic bonding associated with positive entropy change due to the breaking of water structure (Rohatgi and Mukherjee (1971)). The thermodynamics of protein stabilisation also shows the similar nature involving the hydrophobic bonds (Kauzmann (1959)). The positive entropy effect also favours the micelle formations in long chain molecules.
like soaps and detergents (Mukherjee and Ray (1963), Stainby and Alexander (1950), Bruning and Holtzer (1961)). In the dimerisation of rhodamine B and sodium fluorescein it has been suggested by Rohatgi and Singhal (1965, 1966) that due to the positive entropy and low enthalpy change the aggregation in rhodamine B is entropy oriented phenomenon brought about by hydrophobic bonding whereas in sodium fluorescein hydrogen bonding may be responsible for aggregation. Strong hyperchromism was observed on dimerisation of the dyes rhodamine B and sodium fluorescein (Rohatgi (1968)). The aggregation among dye molecules occupying the adjacent sites on the polyanions exhibiting metachromatic spectral shift in solution also involves the hydrophobic bonds and the phenomenon is entropy directed (Mukherjee and Ghosh (1963), Mukherjee and Ray (1963)). An increase in the hydrophobic elements of a solvent increases its ability to disrupt metachromasia (Pal (1965)).

With this aggregation theory as an accepted model for the metachromasia, the report by Sylvén (1954) that metachromasia in a dye like methylene blue could be induced by mercuric chloride, appeared curious. Mercuric chloride is not even a small polyanion, neither it is salt like in nature. Apparently it seemed that the color change in the dye could be due to the formation of coordination complex with mercuric chloride. Since mercuric chloride gives red precipitates and not solution when
mixed with the dye solution, further study of the system could not be pursued. When we thought of studying the chemistry involved in the metachromatic interaction of methylene blue – mercuric chloride, there was no published work in this line except for the report of Sylven (1954). The difficulty was solved by the observation that when an excess of mercuric chloride is added to the dilute aqueous solution of the dye, metachromatic color change takes place immediately without immediate precipitation and also the spectrum of this solution is similar to the reflectance spectrum of the precipitate of dye-mercuric chloride obtained when a limited amount of mercuric chloride is added to the dye solution. Since the dye-mercuric chloride compound stays in solution in the presence of an excess of the inorganic salt, the visible and the ultraviolet spectra of the system could easily be read. On the other hand the formation of immediate metachromatically colored precipitate with less amount of \( \text{HgCl}_2 \) added was the basis for the determination of the stoichiometry of the compound of dye-mercuric chloride; the precipitation of the dye-mercuric chloride compound was further facilitated in the presence of dilute hydrochloric acid, which did not disturb the metachromasia. Analytically the composition of a compound precipitated by mixing a solution containing dye with a solution containing mercuric chloride has been determined by estimating either the dye left in the supernatant solution after centrifuging out the compound formed in the presence of excess dye or by estimating
the mercuric chloride left in the supernatant liquid when the starting solution contains an excess of the inorganic salt; the solvent has been water or 0.4 N HCl. Stoichiometry has also been estimated by titrating 3.26 x 10^{-3} M dye solution conductometrically with HgCl₂ solution either in water or in 1.00 x 10^{-3} N or 3.00 x 10^{-3} N HCl. Results are summarised in the Tables-II and IIIA. It is apparent that the dye/HgCl₂ stoichiometry of the compound formed varies between 1 to 2, excess dye or HCl facilitates the stoichiometry of 2, whereas with water as the solvent or an excess of HgCl₂ the dye/HgCl₂ of the compound approaches 1. The chloride left in the supernatant has also been estimated by titrating with standard silver nitrate solution conductometrically and the result shows that methylene blue chloride, and not methylene blue cation, forms compounds with mercuric chloride. So the composition of the metachromatic red colored compound formed when an excess of mercuric chloride is added to a dilute aqueous solution of the dye, say, methylene blue chloride is \( \text{Methylene blue chloride} \cdot \text{HgCl}_2 \); whereas in the presence of an excess of the dye or in the presence of hydrochloric acid, the composition is \( \text{Methylene blue chloride} \cdot 2 \cdot \text{HgCl}_2 \).

The chromotropic ability of mercuric chloride is similar to the chromotropic abilities of the polyanions. Mercuric chloride induces hypsochromic and hypochromic spectral shifts in metachromatic dyes like methylene blue, toluidine blue, thionine and
acridine orange. Fig. 6 shows that mercuric chloride induced metachromatic spectral shift in the dye methylene blue is more hypsochromic as well as hypochromic than the spectral shift induced by heparin as the chromotrope. Fig. 7 also shows the similar effect of mercuric chloride as chromotrope on the metachromatic dye toluidine blue. Figs. 8 and 9 shows the metachromatic spectral shifts induced by mercuric chloride in thionine and acridine orange respectively. Mercuric chloride fails to induce a sharp absorption band in crystal violet but the dye has its $\alpha -$ band depressed and enhanced absorbances at the shorter wavelength region of the visible absorption spectrum (Fig. 10). Non-metachromatic dyes rhodamine 6G and capri blue are also non-metachromatic in presence of mercuric chloride as shown in Figs. 13 and 14 respectively. It seems that chromotropes are responsible in creating suitable conditions for the dyes to be metachromatic, but it is absolutely the nature of the dyes which determines whether they will undergo metachromatic spectral shifts under such conditions.

Mercuric chloride, though an inorganic salt, is primarily a covalent compound and exists as tetra- or hexa-coordinated complex. The structure of tetra-coordinated mercuric chloride is as follows (Grdenic (1965)):

$$
\text{Cl} \quad \text{Cl} \quad \text{Cl} \quad \text{Cl} \quad \text{Cl} \\
\text{Hg} \quad \text{Hg} \quad \text{Hg} \quad \text{Hg} \quad \text{Hg} \\
\text{Cl} \quad \text{Cl} \quad \text{Cl} \quad \text{Cl} \quad \text{Cl}
$$
Mercuric chloride like iodine, has been used as mordant in Gram staining (Pearse (1960)). According to Basu, Biswas and Pal (1970) when acting as a mordant in Gram staining iodine forms donor-acceptor complexes with crystal violet bound at the surface of the bacteria. Mercuric chloride also forms donor-acceptor complexes with amines having the compositions 1 amine : 1 HgCl₂ and 2 amine : 1 HgCl₂ (Wirth and Davidson (1964)). In such complexes mercuric chloride acts as acceptor of electron donated by nitrogen of the amines. Aromatic amines can form donor-acceptor complexes by donating the electron from the lone pair electrons of nitrogen (Beaumont and Davis (1967), Chandra and Mukherjee (1964)). Alkyl substituted amines also act as electron donors in forming donor-acceptor complexes with p-benzoquinone (Sur 1972). Aluminium chloride can also act as electron acceptor in forming donor-acceptor complexes with suitable electron donating organic molecules (Costanzo and Jurinski (1967)). It has been suggested by Yamane and Davidson (1961) that mercuric ions are bound to DNA predominantly to the pyridine and pyrimidine bases through the lone pair electrons of the nitrogen atoms. Rahn, Landry, Ballista and Setlow (1970) have reported that Hg⁺⁺ at low concentration binds to thymidyl residues of DNA, at high concentration, however, Hg⁺⁺ also binds to other bases.

It is apparent from the structures of all the dyes studied in this dissertation that all of them have terminal amino or
substituted amino groups with a lone pair of electrons on the nitrogens. In this respect dye molecules are similar to aniline or N-substituted anilines. Fig. 15 A shows that aniline has an absorption band at 280 nm. The lone pair orbital of nitrogen in aniline, which is $\pi$ in character, is in conjugation with the aromatic ring $\pi$ system. This conjugation is responsible for the shift of the lowest $\pi \rightarrow \pi^*$ transition from 255 nm (as in benzene) to 280 nm. The difference curve 15 B has a minimum at 280 nm which indicates that the lone pair electrons of the amino-nitrogen are involved in the formation of complex between amine and mercuric chloride. The nitrogen lone pair electrons are not present in anilinium chloride and hence no absorption peak has been observed at 280 nm in such a case as shown by the curve A in Fig. 16. The difference curve 16 B does not have any well defined shape like the difference curve 15 B in the Fig. 15. It is apparent from Figs. 17-21 that the shapes of the difference curves of the dyes methylene blue, acridine orange, crystal violet, rhodamine 6G and capri blue in presence of mercuric chloride are similar to the difference curves obtained with aniline in place of dyes, the difference curves have minima at the ultraviolet absorption peaks of the respective dyes and the higher values for the differences at the shorter and longer wavelengths of these minima. So it is possible that dye molecules are bound to mercuric chloride by partially donating electron from the lone pair electrons of its amino-nitrogen which is
Wirth and Davidson (1964) concludes that aniline, with an unshared pair of π electrons, shows no special affinity for Hg²⁺ as compared to other nitrogen bases with 6-electron pairs. The ring nitrogen or the sulfur atoms of the dye may also be involved in the complex formation with mercuric chloride through their lone pair electrons. The dyes methylene blue, thionine, toluidine blue, capri blue have ring nitrogen and ring sulfur or oxygen atoms, whereas crystal violet does not have either of them. Crystal violet has a third substituted amino group. Similarly rhodamine 6G can form complex through one terminal amino group or through the ring oxygen. It is to be pointed out that though the ultraviolet difference spectra (Figs. 17-21) have all minima at the respective positions of the ultraviolet absorption maxima of the dyes, only in case of the two non-metachromatic dyes capri blue and rhodamine 6G as well as in crystal violet Δ absorbance is never negative.

From the study of the low-frequency infrared spectra of the complex of pyridine and mercuric chloride Coates and Ridlay (1964) conclude that all the chlorine atoms in the complex should be regarded as being bridged rather than in terminal positions, in other words supporting the view that the complex is a polymer held together by chlorine; the complex has a single band at 418 cm⁻¹ where coordinated pyridine commonly absorbs. Dunitz (1957) regards the structures to be a distorted octahedral environment.
Fig. 37 shows the model for the metachromatic dye-mercuric chloride complex that we propose remembering the following facts: (i) mercuric chloride can exist as tetra- and hexa-coordinated complex; (ii) methylene blue-mercuric chloride complex in aqueous solution has stoichiometry of one mole of methylene blue chloride per mole of mercuric chloride in presence of excess of mercuric chloride and (iii) the dye molecules are bonded to mercuric chloride by partial donation of the lone pair electrons of the terminal nitrogen. The metachromatic dye molecules prefer to occupy adjacent sites on the chromotrope so that the interactions between bound dye molecules are possible which will further stabilize the system as well as cause metachromatic spectral shift in potentially metachromatic dyes. Pal and Schubert (1962, 1961a) report that in presence of a moderate excess of chromotrope the metachromatic dye cations saturate as many chromotrope molecules as possible leaving others almost free of dye. In the model in Fig. 37 dye molecules are bound at either side of the tetra-coordinated mercuric chloride chain and the interactions between the adjacent dye molecules will cause the hypsochromic spectral shift in the dye. Such interactions also occur when dye cations are bound to polyanion chromotropes (Bradley and Wolf (1959)). The bindings of dye molecules to the alternate Hg in the tetra-coordinated mercuric chloride chain in presence of excess mercuric chloride are possibly due to the fact that the dye molecules will
suffer steric hindrance and will prefer to coordinate with alternate Hg. This happens also with polyanions of high charge density; in the presence of excess chromotrope the dye cations occupy anionic sites of the chromotrope with some gaps and exhibit metachromasia; in presence of excess dye, however, all the anionic sites of the chromotropes are occupied by the dye cations (Pal and Biswas (in preparation)). In the presence of excess dye the methylene blue-mercuric chloride complex has the composition 2 methylene blue chloride : 1 mercuric chloride, in such a case all the mercury will be hexa-coordinated instead of alternate mercury as shown in Fig. 37. In the dye-mercuric chloride complex the dye molecules will be more closely placed compared to when they are bound to chromotropes like chondroitin sulfate, heparin etc. This is why the mercuric chloride induced metachromatic spectra of methylene blue is more hypsochromic as compared to the metachromasia induced by heparin. It has been observed by Pal and Biswas (in preparation) that on further sulfonation of chondroitin sulfate, a chromotrope with higher charge density is obtained which induces metachromasia in methylene blue which is more hypsochromic and hypochromic as compared to that induced by the parent chromotrope chondroitin sulfate.

In the model in Fig. 37 the aggregation of dye molecules could be extended indefinitely as indicated by the dotted lines at one end, though the stacking of about three-four dye molecules at each side could be sufficient for the metachromatic spectral
shift observed. Pal and Buss (in preparation) have calculated with the free electron gas model and the extended dipole model that trimerisation with a distance of 4.0 Å between adjacent neighbours causes a blue shift of about 100 nm in the absorption spectrum of methylene blue. The position of the chlorine directly attached to the nitrogen of each dye is not precise and is meant to indicate that each dye cation will be associated with its chloride ion in the mercuric chloride-dye chloride complex, as is expected from the composition of the complex. If the coordination is through the lone pair electrons of the ring nitrogen or sulfur atoms of the dye, the stacking model is still valid; only the relative orientation of the stacked dye molecules with respect to the mercury-mercury line will be changed. At present we do not have any data to make any precise statement regarding the site of binding.

The main point that we really want to emphasise in this model is that the metachromasia induced by in suitable dyes by mercuric chloride can reasonably be interpreted by the aggregation theory. The effects of the disrupting reagents like ethanol, sodium chloride etc. are qualitatively similar on the metachromasia induced by mercuric chloride and polyanionic chromotropes. But the strongest support to the aggregation theory as proposed in the model (Fig. 37) comes from effects of mercuric chloride on the spectrum of a mixture of two metachromatic dyes. If the metachromasia of a dye induced by mercuric chloride be due to formation of coordination
complex of dye-mercuric chloride then the observed spectrum of a mixture of the two dyes, say, methylene blue and acridine orange in the presence of mercuric chloride would be as expected from the individual metachromatic spectrum of the respective dyes induced by the inorganic salt; however, the experimental spectrum is strikingly different from the expected spectrum as depicted in the Fig. 38. The curve E is the difference from the experimental and the expected spectrum (sum of curves A and B) of the two dyes methylene blue and acridine orange in the presence of mercuric chloride, the difference spectrum has a positive peak at wavelength somewhat longer than the metachromatic peak of the dye acridine orange and a negative peak around the position of the metachromatic band of the partner dye methylene blue. This is exactly what has been observed with the polyanions \( \lambda \)-carrageenan and chondroitin sulfate as the chromotropes (Pal and Schubert (1963)). This result not only supports the aggregation theory as the interpretation of the mercuric chloride induced metachromasia, but also shows that in the presence of mercuric chloride the dyes are aggregated beyond the dimeric states. Formation of mixed dimers would have positive and negative peaks at longer wavelengths around the \( \beta \)-bands of the respective dyes (Pal and Schubert (1963), West and Pearce (1965), Kuhn and Möbius (1971), Booij and Loeven (1954), Levasin and Suvorov (1958)). The pairs of dyes thionine/acridine orange and toluidine blue/acridine orange behave similarly in the presence of mercuric chloride. It is the criterion of the formation of a
mixed aggregate of a pair of dyes that the absorbance of the dye having band at shorter wavelength will be increased at the expense of the absorbance of the other partner dye. This is what is expected theoretically. When two oscillators of different frequencies are coupled the oscillator strength of the high frequency oscillator increases and that of the other is reduced with some changes in the frequencies of oscillations (Pal and Buss (unpublished data)).

Fig. 39 represents mixed metachromasia of methylene blue and crystal violet induced by mercuric chloride. With polyanion as chromotrope, methylene blue and crystal violet show only homo-aggregations (Pal and Schubert (1963)), and crystal violet gives only poor metachromasia with mercuric chloride as the chromotrope. Yet the difference spectrum (Fig. 39 E) shows positive deviation at shorter wavelength and negative deviation at longer wavelength region of the spectrum. In this figure the positive peak is, as expected, around the metachromatic band of crystal violet that is observed with a good chromotrope (Stork et al (1973)), but the negative peak is not at the metachromatic band of the partner dye due to the close proximity of the \( \mu \) - band of the two dyes and due to the weak metachromasia of crystal violet induced by mercuric chloride (Fig. 10). This shows that the mixed metachromasia of crystal violet with methylene blue is stronger than the individual metachromasia of crystal violet alone in the presence of mercuric
chloride. This is the usual observation in mixed metachromasia (Pal and Schubert (1963)). Induction of mixed metachromasia by mercuric chloride indeed supports the proposed model (Fig. 37).

B. Acidified tungstate as chromotrope:

Sodium tungstate normally does not act as a chromotrope in inducing metachromatic color changes in dyes, but when acidified with dilute acids tungstate induces metachromasia in the potentially metachromatic dyes (Figs. 25-29). The chromotropic character of acidified tungstate is however weaker than that of heparin or mercuric chloride as is shown in the Fig. 25, where the tungstate induced metachromasia is shown to be the least hypsochromic. This is also apparent visually as the tungstate induced metachromatic color in, say, methylene blue is less reddish than when induced by mercuric chloride.

The tungstate ion $\text{WO}_4^{2-}$ in sodium tungstate has been shown to be tetrahedral in nature by Raman spectroscopy in alkaline and neutral solutions (Woodward and Roberts (1956), Busey and Keller (1964)). On acidification, when pH drops below 7, polytungstate ions are formed and the exact nature of these intermediate polytungstate ions has not been yet settled and leads to the disagreement among different workers. However, the subject has been
extensively reviewed by Kepert (1962). From an acidified tungstate solution paratungstate, metatungstate or hydrated tungstic oxide may result in which octahedral coordination prevails (Freemann (1959,1963)). It has been suggested that polymerisation proceeds via di- and tri- tungstates (Jain and Dogra (1968), Pan, Liu, Sheng and Hseu (1953)). Among these soluble anions only the metatungstate exists in solution in a sufficiently stable condition at pH 3 which can be isolated as the free acid (Buchholz (1940)).

The stoichiometry of the interaction of acidified tungstate and a dye could not be established analytically due to the lack of a suitable method of estimation of residual tungstate in the presence of excess dye. Stoichiometry was estimated only conductometrically (Figs. 23 and 24 and Table V). In the conductometric titrations of 2.00 x 10^-1 M methylene blue by 1.00 x 10^-1 M sodium tungstate acidified by different concentrations of HCl (zero to 0.05M), in every case there is one break corresponding to a dye/tungstate, mole/mole ratio 1.0 and a second break occurs with the value of this ratio having value \( \approx 0.6 \pm 0.04 \). In case of 0.1 N and 0.2 N HCl as solvents for the tungstate (Figs. 23A, 23B), there are also breaks corresponding to dye/WO_4^- ratio 2.0. It is known that tungstate forms various polytungstates on acidification (Kepert (1962)) and the attainment of equilibrium is sometimes a very slow process. Spitsyn and Pirogova (1957) measured the dialysis constant immediately after acidification as well as
after equilibration and found the average molecular weight of the species was greater immediately after acidification showing the formation of unstable high molecular weight species. The type (s) of the polytungstate formed also depends upon the ratio \( \text{H}^+/\text{WO}_4^- \) in the starting solution. Since tungstate induce metachromasia only upon acidification, it can be said reasonably that it is the polytungstate which actually acts as the chromotrope. On the other hand since the acidity of a tungstate solution can be varied over some range (e.g. 0.05 to 0.15 M HCl as the solvent form tungstate) to induce the chromotropic character, it is difficult to assign the chromotropic ability to a specific polytungstate. From the Figs. 23 and 24 it is apparent that the average charge per tungsten atom of a polytungstate depends on the acid to tungstate ratio in the starting solution. When the concentration of added hydrochloric acid in the solvent is below 0.05 M, 0.6 dye cations bind per tungsten atom in the presence of an excess of tungstate, this ratio changes to 1 when there is a limited amount of tungstate. Iguchi (1956) measured a charge of 0.55 per tungsten atom for a fourteen days-equilibrated tungstate solution at pH below 6. In the absence of any excess tungstate, one dye cation is bound per tungsten atom, with hydrochloric acid of higher concentrations (0.1 M and higher) the initial break in the titration curve corresponds to a dye/W ratio of 2. Since the chromotropic ability of tungstate is not very sensitive to the concentration of hydrochloric acid to be added in the mixture
(or rather to the $\text{H}^+ / \text{WO}_4^{-}$ ratio), it is not possible to assign the metachromasia to a particular dye/$W$ ratio. On the other hand since normal tungstate fails to act as a chromotrope and since acidification results in the formation of polytungstates, the chromotropic ability of tungstate can reasonably be assigned to polytungstate $(s)$. The dye cations may be bound to the polytungstate ion $(s)$ with dye/$W$ ratio dependent on the composition of the starting solution regarding tungstate and $\text{H}^+$. From the analysis of the chloride in the supernatant liquid after the dye-acidified tungstate precipitates have been centrifuged out, it has been found that methylene blue cation binds with polytungstate ion, not the methylene blue chloride (Table-VI). Metachromasia of methylene blue in presence of acidified tungstate is completely destroyed by ethanol while even saturated aqueous solution of sodium chloride fails to do so. The dye cations thus bound will suffer stacking and hence metachromatic spectral shifts. With such a model in view, one would naturally expect induction of mixed metachromasia (or heteroaggregation) in suitable pairs of dyes by acidified tungstate. Acidified tungstate, in fact, induces mixed metachromasia as is evident from the Figs. 40 and 41. The nature of the difference curve (Fig. 40 E) is comparable with the corresponding figure with mercuric chloride as the chromotrope (Fig. 38 E) or with $\lambda$-carrageenan as the chromotrope (Pal and Schubert (1963)). Fig. 41 also shows the formation of mixed aggregates of
acridine orange and crystal violet in the presence of acidified tungstate.

It is apparent from the above discussion that, in contrary to the apparent apprehension, the metachromasia induced in potentially metachromatic dyes by salts like mercuric chloride or tungstate can reasonably be interpreted by the aggregation theory.