polarographic half-wave potential, while not having a corresponding effect on the potential of a stationary electrode, to cause the difference observed between the polarographically and potentiometrically determined values of the complexity constants.

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[CONTRIBUTION FROM THE STUDY GROUP ON RHEUMATIC DISEASES, AND THE DEPARTMENTS OF CHEMISTRY AND MEDICINE, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

A Qualitative Theory of Metachromasy in Solution[1]

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A theory is proposed to account for the production of metachromatic colors in solutions of anionic mucopolysaccharides or anionic detergents. It is based on four points: (1) that chromotropes exist in solution as anionic clusters or micelles, (2) that cations are reversibly bound within such micelles, and are bound more firmly the higher the cationic charge, (3) that metachromatic dyes exist in solution in a set of equilibria among polymeric dye ions with different charges, (4) that the chromotropic micelle selectively and reversibly binds the dye ion of highest charge and so causes a shift in the equilibrium of dye ions in the intermicellar space. Some new experimental data in support of this theory are found in a detailed examination of the deviations from Beer's law of several dyes. The theory is able to make some predictions on whose verification its validity will depend.

Previous studies from this Laboratory[2-4] as well as from others[5-7] provide a collection of data which with further data here presented makes possible a theory of metachromasy limited to aqueous solutions. The theory is therefore not directly applicable to the conditions under which tissues are stained for histological purposes. Though it is only qualitative, it should be possible to develop it quantitatively when further information is available.

The theory consists of four postulates, parts of which have been proposed by earlier workers. (1) Chromotropes exist in solution as anionic globular clusters or micelles whose charge density depends on the nature of the chromotrope and the solution environment. These micelles are separated by intermicellar spaces of small anion density. (2) Cations are reversibly, and more or less firmly, bound within such anionic clusters or micelles. The relative firmness of binding of different cations increases with their charge. (3) In solutions of metachromatic dyes there is a set of equilibria among several polymers of the cation represented by the equations,

$$D^{+} \rightleftharpoons D_2^{++} \rightleftharpoons D_3^{+++} \rightleftharpoons D_4^{++++} \rightleftharpoons \qquad (I)$$

The proportions of the several molecular species will depend on the dye concentration. Each molecular species has its own characteristic absorption spectrum. (4) The metachromatic color is produced as a result of the selective and reversible binding within the anionic cluster, of the polymeric dye cation of highest charge available. Binding of the dye cation of high charge causes a shift in the equilibria (I) with a drop in the concentration of ions of lower charge. The effect is that even in dilute dye solutions the concentration of dye cations of high charge is increased in the total micellar and intermicellar space. Experimental data in support of each of these postulates will now be discussed.

Chromotropes are of two kinds: anionic polyelectrolytes of high molecular weight such as chondroitin sulfate, heparin, hyaluronate, alginate, silicates and metaphosphates and anionic detergents. Work of Ogston and Stanier[8] and of Blumberg and Oster[9] shows that hyaluronate exists in solution as spherical particles of molecular weight of the order of 10^7. The same particle shape is assumed by Fuoss and Strauss[10] to account for the viscosity properties of synthetic linear polyelectrolytes. Detergents have been assumed to exist in solutions as micelles.[11] Thus the first postulate extends to all chromotropes a property which has been found for some and is generally assumed for others.

That cations become reversibly bound within charged globular micelles is supported by experimental data on the properties of Fuoss clusters[12] and Hartley micelles.[13] That the firmness of binding of cations within such micelles should increase with the cationic charge is an extension of this idea. The dependence of firmness of binding on cationic charge is a phenomenon analogous to the rule of Schulze and Hardy in the flocculation of hydrophobic colloids[14] and to the reversal of charge produced in anionic polyelectrolytes by cations of different valence.[15] The second postulate is merely a statement of the behavior that would be expected of cations in a solution containing anionic micelles. Such binding can lead to precipitation particularly with cations of high charge. This occurs when lysozyme or protamine is added to solutions of chondroitin sulfate, heparin or hyaluronate, or when dye

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(14) J. T. G. Overbeek, ref. 13, Vol. 1, 1952, p. 81.

(15) H. G. Bungenberg de Jong, ref. 13, Vol. 2, 1949, p. 259.

and chromotrope reach a sufficiently high concentration.

The third postulate is a statement explaining why some dyes deviate from Beer's law.[16] While deviation from Beer's law has been explained by dye polymerization, there are many dyes that polymerize and yet conform to Beer's law. A structural principle that distinguishes these two classes of dyes seems to be expressible in terms of the location of the permanent charge of the dye ion. In those dyes that conform to Beer's law the ionic charge is not part of the chromophoric system whereas in those that do not conform to Beer's law the charge is part of the chromophoric system. To the first class belong dyes such as orange G and congo red, in which the ionic charge has an assignable location. To the second class belong dyes such as methylene blue, crystal violet and eosin, dyes in which the ionic charge cannot be assigned to a particular location.

For those dyes in which polymerization is thought to account for deviations from Beer's law, the assumption is made that the ion species at each successive stage of polymerization has its own characteristic absorption spectrum. An attempt to work out quantitatively the relative concentrations of monomeric and dimeric forms with variation in total dye concentration was made by Rabinowitch and Epstein[17] in the cases of two thiazine dyes. The fundamental data on which the theory of these workers was based consisted of extinction coefficients measured at only four dye concentrations. More extensive measurements here presented show the need for a more elaborate theoretical treatment to account for the extended data. At high concentrations of methylene blue both the α- and β-bands drop and so according to Rabinowitch and Epstein both the monomeric and dimeric forms are disappearing. Taking a cue from the existence of a μ-band in the metachromatic spectra,[13] the extinction coefficient at the wave length of the μ-band has also been measured as a function of dye concentration. The rise in this band with dye concentration (Fig. 4) is consistent with its representing the peak in the absorption spectrum of a polymeric form of the dye cation. Whether this polymer is a trimer formed by combination of monomer and dimer, or a tetramer formed by combination of two dimers, is not now clear. The existence of such highly charged cations has sometimes seemed doubtful. Michaelis,[18] for instance, said methyl green obeyed Beer's law in contrast to other triphenylmethane dyes because of the high repulsive force between two doubly charged cations. A study of methyl green included in the present work shows that it does not conform to Beer's law.

The fourth postulate is a natural consequence of the first three. It accounts for the metachromatic color changes of methylene blue and crystal violet, the two dyes most carefully studied. In the presence of appropriate amounts of chromotrope there is a depression of both α and β-bands and a rise in the μ- or metachromatic band. The amount of chromotrope that produces the maximum change in these bands is in the neighborhood of an amount equivalent to that of the dye. The production of metachromatic color can be reversed by the addition of a neutral salt which supplies a cation in the intermicellar space. This can compete with the intramicellar dye cation. The effectiveness of a neutral salt in suppressing metachromasy is greater the higher the charge of its cation as shown by Bank and Bungenberg de Jong.[6] Increasing chromotrope concentration may also depress metachromasy because the intermicellar cation concentration is increased. If the chromotrope is introduced as its calcium salt, the stronger competing effect of the calcium ion is shown by the markedly lower effect produced and its quicker disappearance on raising the chromotrope concentration.[2] Consistent with this theory is the fact that at higher dye concentration, potassium chloride, in the concentrations used, did not change the metachromatic to the normal spectra of methylene blue or crystal violet in the presence of chondroitin sulfate. Neither did higher chromotrope concentration cause reversion of the metachromatic to the normal spectra as it did at lower dye concentration.[4] At high dye concentration the intermicellar salt cation cannot be raised sufficiently to drive out intramicellar dye. At the salt concentrations used, below $0.1M$, no effect is produced on the equilibria (I) as is apparent from the absence of effect on dye spectra.

Experimental and Results

The new data consist of a more precise study of the absorption spectra of methylene blue, crystal violet and methyl green as a function of dye concentration. The dyes were recrystallized from water and when developed chromatographically on paper, each moved as a single spot. At low dye concentrations (below 10^{-5}) there is difficulty in getting reproducible density readings as has been pointed out before,[2] apparently due to adsorption of dye to the silica cell. Experiments at a dye concentration of about 10^{-4} M showed that in the case of methylene blue addition of HCl to a concentration of 0.1 M produced no change in the absorption curve. At lower dye concentration such addition of HCl gave far more reproducible results and generally higher optical densities. With crystal violet and methyl green, HCl could not be used since these dyes change color in acid so addition of sodium chloride was tried instead. While NaCl at concentrations up to 0.1 M has no effect on the absorption spectra of these dyes, it may also have no effect on their adsorption to silica. It is therefore felt that the spectra and extinction values of crystal violet and methyl green below 10^{-5} may not be valid. This uncertainty cannot be overcome at present. Fortunately our interest is mainly centered in the more concentrated dye solutions and in the case of methylene blue there seems to be no reason to question any part of the curves.

The results are summarized in the diagrams in which the ordinate units are molar extinction coefficients, E_M and $E_M c_M l = \log I/I_0 =$ optical density. In this equation c_M is the molar concentration of dye in solution and l is the length of light path through the solution. Silica cells were used which had light paths 0.01, 0.1, 1.0 or 10.0 cm. All measurements of optical density ($\log I/I_0$) were made with a Beckman DU spectrophotometer at a temperature of 20 to 25°. Successive dilutions were made from the solution of highest concentration by addition of 20 ml. of this solution to 10 ml. of solvent. For methylene blue the solvent was 0.1 M HCl in water, for crystal violet and methyl green 0.01 M sodium chloride was used.

Figures 1 and 2 show the absorption spectra of methylene blue and crystal violet at a series of concentrations. For each dye, maxima or shoulders appear at only two wave lengths corresponding to α- and β-bands. In contrast to Fig. 2 is Fig. 3 in which are shown absorption spectra of

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[16] S. E. Sheppard, *Rev. Mod. Phys.*, **14**, 303 (1942).

[17] E. Rabinowitch and L. F. Epstein, THIS JOURNAL, **63**, 69 (1941).

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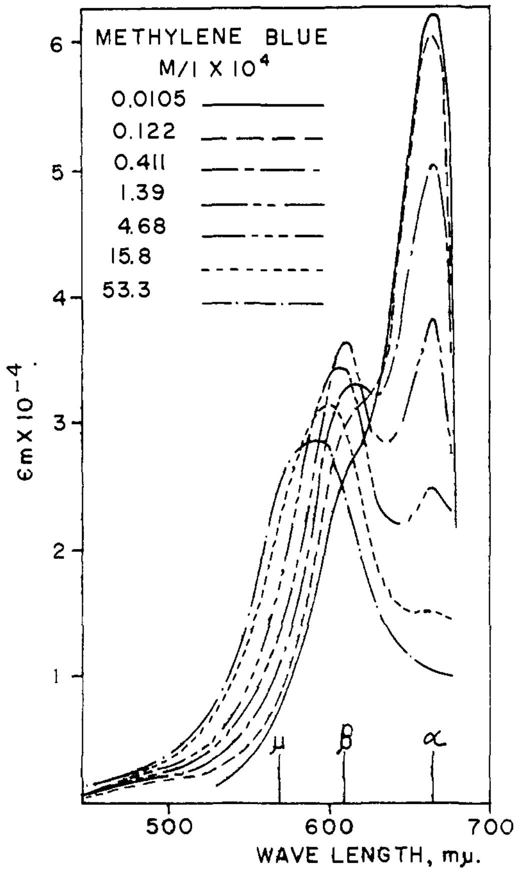

Fig. 1.—Absorption spectra at several concentrations of methylene blue. Arrows indicate wave lengths at which the α-, β- and μ-bands occur.

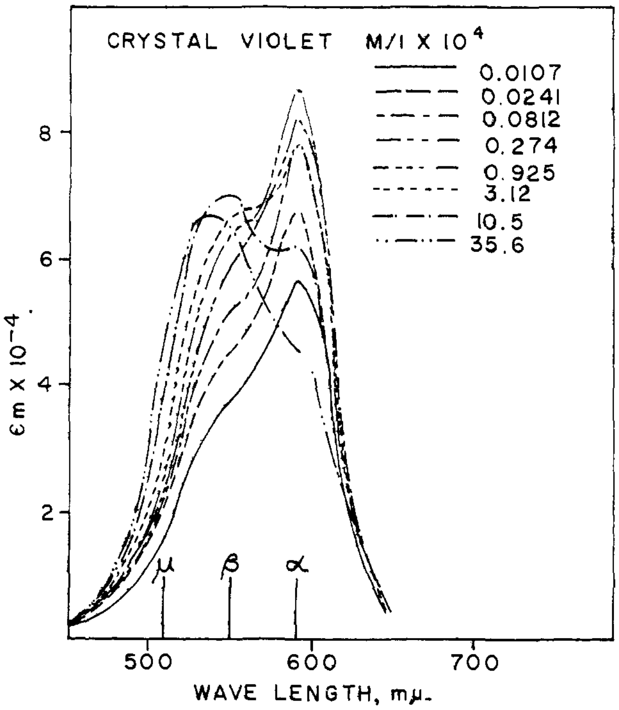

Fig. 2.—Absorption spectra at several concentrations of crystal violet. Arrows indicate wave lengths at which the α-, β- and μ-bands occur.

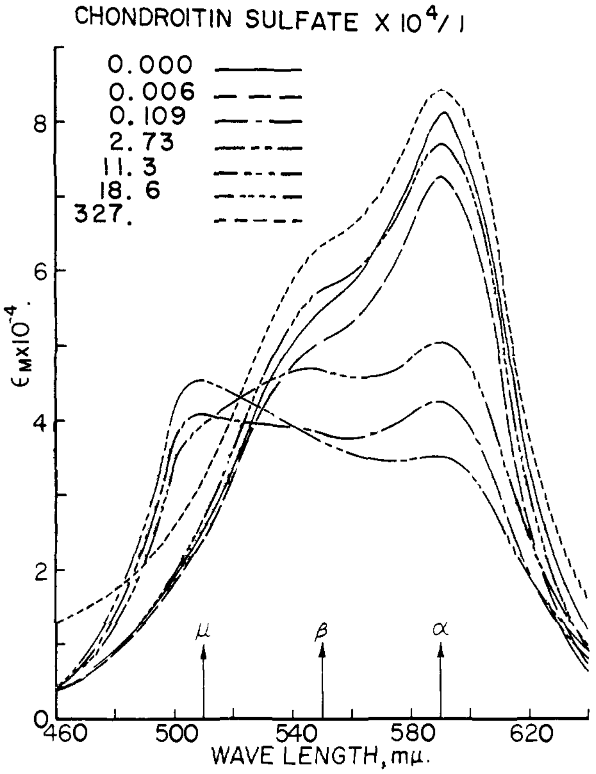

Fig. 3.—Absorption spectra of crystal violet (1.25 × 10^{-5} M) at several concentrations of chondroitin sulfate. Arrows indicate wave lengths at which the α-, β- and μ-bands occur.

crystal violet at one dye concentration and a series of chondroitin sulfate concentrations. Maxima appear not only at the α- and β-positions but a new band appears at a shorter wave length. Corresponding in all respects to Fig. 3 is the set of curves for methylene blue previously published.[2] Koizumi and Mataga[19] have also pointed out, for pyronine G, that the set of spectra at varied dye concentration shows bands at only two wave lengths while the metachromatic spectra at varied polyvinyl sulfate concentration show bands at three wave lengths. Although the μ-bands do not appear in Figs. 1 and 2 it is clear that the absorption increases with dye concentration at this wave length. This appears explicitly in Figs. 4 and 5 in which the extinction coefficients of the α-, β- and μ-bands are plotted against dye concentration over a range of nearly four log units. The important new feature now emerges that for both dyes at high concentrations, both the α- and β-bands are falling with increasing concentration while the μ-band is rising. Thus the directions in which these bands change are the same as occurs in the presence of increasing concentration of chromotrope at low chromotrope concentration. The ultraviolet bands, plotted on the same figures, are not discussed in this paper since the theory presented is confined to the visible bands. Changes in the ultraviolet bands are generally less pronounced than changes in the visible bands.

In order to select the proper wave lengths to be plotted for methyl green, the molar extinctions of the dye at several concentrations were plotted against wave length. A single band appears at 630 mμ. A suggestion of a second or μ-band appears as a slight shoulder at 590 mμ when absorption spectra are plotted at a fixed concentration of methyl green and at several concentrations of chondroitin sulfate. In Fig. 6, the log concentration of methyl green is plotted against molar extinction values. The band at 630 mμ drops off very sharply at high dye concentration (10^{-2} M). In contrast to this, the extinction at 590 mμ, at concentrations above 10^{-3} M is constant. It might be assumed that the

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[19] M. Koizumi and N. Mataga, THIS JOURNAL, **76**, 614 (1954).

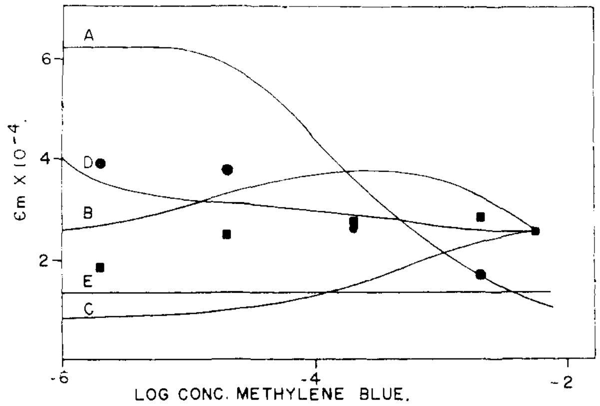

Fig. 4.—Molar extinction values of methylene blue in 0.1 *N* HCl at the α-, β- and μ-bands and those wave lengths where maxima occur in the ultraviolet, plotted against molar concentrations of methylene blue: *A*, α-band, 665 mμ; B, β-band, 610 mμ; C, μ-band, 570 mμ; D, 290 mμ; E, 245 mμ. For comparison, the data of Rabinowitch and Epstein[17] are plotted on the same curve: ●, 656.5 mμ; ■, 600 mμ.

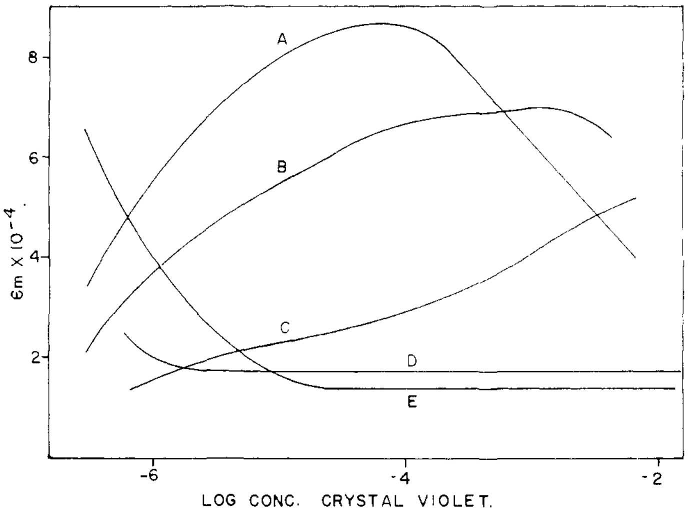

Fig. 5.—Molar extinction values of crystal violet at the α-, β- and μ-bands and those wave lengths where maxima occur in the ultraviolet, plotted against molar concentrations of crystal violet: A, α-band, 590 mμ; B, β-band, 550 mμ; C, μ-band, 510 mμ; D, 300 mμ; E, 250 mμ.

constant extinction at 590 mμ is the result of a rising μ-band balanced by the falling nearby α-band at 630 mμ. Allowing this, the curves of Fig. 6 provide evidence that methyl green, a doubly charged cation, *may* form a dimer at very high dye concentrations. The range of experimental work is limited by the color density of the dye and the sensitivity of the spectrophotometer.

Discussion

The theory presented is partly based on a revision and extension of the work of Rabinowitch and Epstein[17] on methylene blue. Their work has already been criticized by Michaelis and Granick.[7] Furthermore Lemin and Vickerstaff[20] pointed out that the extinction of the β-band did not rise to a limiting value but passed through a maximum and so suggested the existence in solution at high dye concentration of polymers higher than dimers.

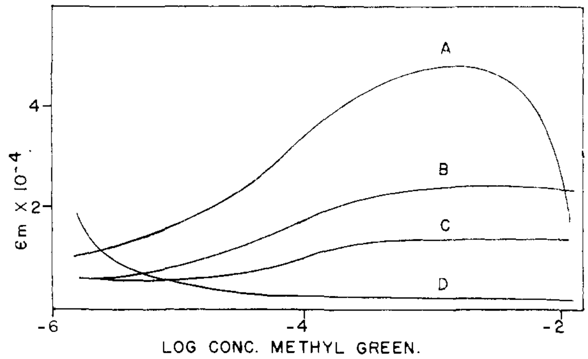

Fig. 6.—Molar extinction values of methyl green at the α- and μ-bands and those wave lengths where maxima occur in the ultraviolet, plotted against molar concentrations of methyl green: A, α-band, 630 mμ; B, μ-band, 590 mμ; C, 310 mμ; D, 240 mμ. Each curve of Figs. 4, 5 and 6 was determined with about 25 points equally spaced along the abscissa.

The present work supports the views of Lemin and Vickerstaff and carries it further by studying the variation in extinction at a wave length where no band explicitly appears at any dye concentration except in the presence of chromotrope. The variation with dye concentration of the extinction at this wave length, the metachromatic or μ-band, is consistent with its representing a higher polymeric form, either a trimer or a tetramer. This idea applies to both methylene blue and crystal violet. The situation with respect to methyl green is different. Here the monomeric form is a divalent cation. Only at very high dye concentrations does this band precipitously drop. Though study of the metachromatic spectra only dimly suggests the existence of a μ-band, Fig. 6 shows that this could represent a dimeric quadrivalent cation.

Since the time of Lison,[5] the thought has been sometimes expressed that ester sulfate groups in a high molecular weight molecule are necessary to produce metachromatic color.[21] Even leaving aside hyaluronate because of its frequent contamination with chondroitin sulfate, there are other substances free of ester sulfate that are chromotropic such as alginate and myristate. Walton and Ricketts[22] reported an interesting study in which they related experimentally the ester sulfate content of a large series of polymers with the extent of precipitation of toluidine blue after shaking with petroleum ether. They then tacitly identified the extent of precipitation with metachromatic activity. Mukerjee and Mysels[23] have reported on the metachromatic effect of sodium lauryl sulfate on pinacynol and related it to the production of an extremely insoluble salt of the dye cation and detergent anion. Spectral changes in pinacyanol are considered a good measure of critical concentration of micelle

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formation. The concern of the present work is restricted to metachromasy produced in solutions in which no concomitant precipitation is apparent.

Recent explanations of the production of metachromatic color have assumed the existence of dye polymers. Michaelis and Granick[7] suggested that in the presence of agar molecular aggregates of dye molecules higher than dimers are formed which are adsorbed by the agar. Wiame[24] thought that when many molecules of dye are joined to the same molecule of hexametaphosphate they can be considered to be polymerized. In the presence of larger amounts of metaphosphate the dye molecules become scattered over the chromotrope and are no longer close enough together to act as though polymerized. Koizumi and Mataga[19] thought that when dye ions are adsorbed to polyvinyl sulfate a new aggregation of dye ions is formed which does not participate in the monomer–dimer equilibrium. Weissman and co-workers[25] thought that metachromasy in the presence of nucleic acids depends on physico-chemical states of the dye and chromotrope, that the dye is linked to chromotrope by polar bonds, and that dye molecules are linked to each other by non-polar bonds. Booij and co-workers[26] thought that the basis of metachromasy is an electrochemical attraction of dye and chromotrope, that larger aggregates of dyes should be regarded as micelles, and that the presence of long polyanionic molecules stimulates the formation of long dye micelles.

The theory presented in the present work allows some predictions to be made and suggests experiments that may select one of the several theories proposed. (1) It would be expected that for any given metachromatic dye the color changes produced by any chromotropes would all be similar. The bands representing monomeric and dimeric forms would always decrease as that representing the next higher form increases. Differences among chromotropes would depend only on the differing extent to which they bind the dye cation of highest charge and this would be largely determined by the anionic density in the chromotrope micelle. On the basis of several of the theories mentioned above a more variable color might be expected depending on the extent of crowding of dye ions. (2) The effectiveness of added salts or higher chromotrope concentration in destroying metachromatic colors depends on the competitive action of their cations. The main controlling factors will therefore be the concentration of salt cation, its charge and the dye concentration. (3) A point not directly concerning metachromasy is raised in the third postulate. In the common cationic dyes the permanent ionic charge is part of the chromophoric system of alternating double bands. The postulate simply records that such dyes deviate from Beer's law. It has now been shown that methyl green also deviates, the significant deviation occurring only at very high dye concentration, near the limit of measurement. It is to be expected that all cationic dyes will show deviations from Beer's law. It is further to be expected that, of anionic dyes, only those such as eosin, will show deviations and this is known to be so. (4) Such anionic dyes may be expected, by the present theory, to be metachromatic in the presence of cationic detergents or cationic polyelectrolytes such as those synthesized by Fuoss and his co-workers.[12] (5) On the basis of the first two points raised above it should be possible to develop a quantitative theory of metachromasy in solution for those dyes which show sufficient variation in α-, β- and μ-bands to allow calculation of the equilibrium constants and identification of the μ polymer. This is not possible on the basis of any other theory yet proposed.

A few words of speculation as to the cause of the color change that occurs on polymerization of cationic dyes might be of interest. The change from normal to metachromatic color is both hypochromic and hypsochromic. Such an effect is observed in the shortening of the conjugated resonance system in linear chromophores.[27] It is unlikely that this effect could operate in the polymerization of thiazine or triphenylmethane dyes. It seems more likely that polymerization could involve interaction of some of the π-electrons of the combining molecules, imposing constraints such that the energy difference between ground and first excited levels is successively greater in dimeric and polymeric forms. A possible origin of such a constraint can be imagined on the basis of the theory of Kuhn,[28] relating structure and absorption in organic dyes. Suppose that when two methylene blue cations dimerize an interaction occurs between the π-electrons in level N/2, the most loosely bound electrons and those responsible for the α-band, so that a constraint is imposed and they effectively cease to be π-electrons. The π-electrons at the next lower level (N/2) − 1, now the most loosely bound, have to make a higher energy jump to reach the first excited state. This means absorption at a shorter wave length (hypsochromic) and with a lower probability of occurrence (hypochromic). On this view only the most loosely bound π-electrons are involved in polymerization so only slight effects would be expected in ultraviolet bands.

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