# Metachromasy: An Experimental and Theoretical Reevaluation*

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ABSTRACT

Non-chromotropic substances such as fibrin and gelatin and most tissue and cellular structures stain orthochromatically with internal dye concentrations of such metachromatic dyes as methylene blue and toluidine blue which, if in solution, would be metachromatic. Therefore, at ordinary levels of staining these substances depress the natural tendency of these dyes to change color. However, at elevated levels of dye-binding **metachromasy** eventually occurs. This phenomenon is explained on the basis of the distribution of dye-binding sites. In these substrates, by contrast with chromotropic substances, many binding sites are too far removed for dye interaction, consequently the interaction frequency can become high enough to produce a color change only as saturation of the available sites is approached.

It is also shown that the destruction of color is a characteristic of metachromasy and that water molecules intercalated between approximated dye ions are responsible for the loss and change of color. A concept of metachromasy is proposed in which the interaction between water molecules and suitably approximated dye ions plays an essential role.

The experimental studies are described against a background of the history and evolution of ideas on metachromasy. The literature is reviewed and reassessed particularly from the physicochemical viewpoint.

**Metachromasy**[1] is a characteristic color change which certain aniline dyes exhibit when bound to particular substances or when concentrated in solution. The phenomenon was originally noticed in tissue sections in which certain components, the so called chromotropes, stained in the metachromatic color. For example, the basic dye toluidine blue becomes distinctly pink when bound to cartilage matrix. A similar but less obvious color change can be seen in aqueous dye solutions under certain concentration and temperature conditions.

Conversely, **orthochromasy** is the relative absence of color change when such a dye is in solution or when it is bound. Although some change in spectral absorption can be anticipated when the dye interacts with a substrate, the change with non-chromotropic substances is usually too small to be seen. Except for incidental references, the possible significance of orthochromasy has been overlooked. Attention has been directed to the more striking phenomenon, metachromasy, and, since the investigations of Lison (1935 *a* and *b*; 1936 *a*), histologists have focused increasingly upon it as a histochemical test for sulfate esters of high molecular weight. However, orthochromasy is the rule in

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[1] The terms metachromasy and metachromasia were selected respectively by Levine and Schubert (1952) and Schubert and Hamerman (1956) as the proper English equivalent of *metachromasie* (German) and *métachromasie* (French). In practice the former is popular in the chemical literature, the latter in the biological literature. Although examples of both forms can be found (Gould, 1894; Lee, 1905) the references to this phenomenon in the English language before 1940 generally employ metachromatism or metachromism. The earliest use known to us (*J. Roy. Micr. Soc.*, 1893, **13,** 563) is actually an editor's abstract of a publication in German. We prefer the term *metachromasy.*

tissue staining, and most tissue elements stain bluish rather than purplish or reddish with metachromatic dyes like methylene blue and toluidine blue. The staining may be rather intense and yet orthochromatic. This is well illustrated by some quantitative spectrophotometric observations on methylene blue bound to films of pure fibrin, a protein whose physicochemical state compares with that of fixed tissue proteins (Ferry and Morrison, 1946; Singer and Morrison, 1948). The dye showed no major shift in absorption upon binding, although the concentration in the film eventually exceeded the level at which aqueous dye solutions develop a metachromatic shift (Singer *et al.*, 1951; Singer, 1954). Therefore, fibrin and, presumably, many tissue proteins suppress the metachromatic tendency of these dyes.

In the present work these initial results are elaborated by spectrophotometric and histochemical studies of two model systems, fibrin and gelatin. Our studies show that as more dye is bound metachromasy gradually develops even in substances which initially suppress the color change. We have also examined the role of water in metachromasy and the relation between color change and reduction of color intensity in agar films, in gelatin films containing heparin or agar, as well as in sections of variously fixed tissue. The results have led us to reevaluate the significance of the phenomenon and to modify and extend existing concepts of the nature and origin of the color shift. We have had the occasion to review the development of these concepts and have been repeatedly impressed with the wealth of information which has been forgotten, ignored, or misinterpreted. Therefore, we are including herewith a survey and evaluation of the development of our knowledge and understanding of metachromasy. Two recent works, of different scope and emphasis than the present one, also serve to review and to interpret the phenomenon of metachromasy (Kelly, 1956; Schubert and Hamerman, 1956).

## HISTORICAL REVIEW

### *The Recognition of Metachromasy; Evolution of Terminology*

It is a common belief that metachromasy, in name and description, originated in the work of Ehrlich. This view neglects the notable works which preceded and accompanied it. Several reports of changes in dye color in histological sections appeared in 1875. These included accurate, fundamental observations and clearly foreshadowed the controversy that developed.

Cornil (1875 *a* to *d*) accurately described the color change of several purified dyes. He conceived of the dye as dissociating, separating, or decomposing into two colors under the influence of certain tissues. The work is outstanding because he recognized the importance of using pure dyes in a study of color change, appreciated the effect of dye concentration, demonstrated that the tissue elements which stain in the altered color have the highest dye affinity, and that this color is more stable in water than in glycerine. Cornil employed the color change to study amyloid degeneration and prophesied that this reaction would be used for "complete histological analysis." His colleague Ranvier (1875) advocated the use of quinoline blue (pp. 102 and 103) because it gave varicolored effects and he used the color change (p. 443) to trace cartilage during bone formation. Jürgens (1875), who also used a purified dye, noticed the color change in many tissues but focused upon amyloid. By contrast, Heschl (1875, 1876), who used a violet ink, asserted that the new color appeared only with amyloid. He ascribed this effect to the presence of a second dye, but achieved only mediocre success with a deliberate mixture.

These four authors recorded the phenomenon of metachromasy, and two initiated opposed explanations, namely that the color change depends upon the interaction of special substrates with a particular dye (Cornil) or that it depends upon the presence in the stain of a second dye which colors the special substrate (Heschl). These concepts were to play a major role in the controversy over the nature of metachromasy and still persist in discussions of specific dyes like methylene blue. It is also easy to sense the initial rumbling of the half-century long controversy over whether staining is a physical or a chemical process (Fischer, p. 181, 1899; Gierke, p. 221, 1884; Griesbach, 1886, 1888; Hoyer, 1890; Mann, p. 368, 1902; Mayer, 1896; Paneth, 1888; Pappenheim, 1901; Schaffer, 1888; Solger, 1888; Unna, 1891; etc.).

Ehrlich, an associate of Heschl, cited Cornil, Ranvier, and Jürgens in his first publication (1877). He reported a "definite reaction color" in goblet cells and in the granules of certain connective tissue cells. In addition, he noticed that with amyloid this color is sensitive to alcohol. This paper has been cited frequently for the first description and the naming of metachromasy. In his unpublished thesis[2] of the follow-

[2] Ehrlich's thesis was not published. Michaelis, who noticed its absence in the bibliographies published after Ehrlich's death, recovered the thesis at Leipzig and recorded (1919) some of its contents. The original could not be located for us but we were able to obtain a photocopy for the Cornell University Library of a transcript preserved at the Georg Speyer Haus in Frankfort. We are pleased to acknowledge our indebtedness to this institution. Since then a translated copy of the thesis has appeared in the Collected Papers of Paul Ehrlich, volume 1, 1956, New York, Pergamon Press.

ing year Ehrlich used the adjective "metachromatic" without definition—as if it were familiar to the reader. Indeed, it already existed in the chemical literature for certain color changes (Ackroyd, 1876 *a*, *b*). Ehrlich, however, has the distinction of using it for the changed color of a dye after combining with tissue. In the dissertation, Ehrlich proposed that staining by aniline dyes consists in forming "atypical double salts." This concept was based on "proof" that a "metachromatic double salt" is formed between dye and the granules in those connective tissue elements which he renamed "mast cells." Ehrlich compared his results on other substances with reports in the literature: amyloid, (Baumgarten, 1876; Cornil, 1875; Heschl, 1876; Ranvier, 1875); cartilage, (Cornil, 1875; Heschl, 1875; Jürgens, 1875); mucus, (Ehrlich, 1877; Schiefferdecker, 1878); and myelin, (Carl, 1877; Treitel, 1876).

Some of these findings were published when (1879 *a*, *b*) Ehrlich introduced the term mast cell into the formal literature. He recommended certain dyes because these stained "*die granulierten Zellen metachromatisch*, that is to say in a shade deviating from the color of the dye employed."

"Chromoleptic substance," although peculiar to Adamkiewicz (1884 *a*, *b*; 1885 *a*, *b*; 1888; 1890), was the first generic term for substances which stain metachromatically.

Paneth (1888) was the first to use the expression "*metachromasie*" and to carefully define it as a separate and distinct phenomenon (see Mayer, 1920, p. 73). He also regarded it as proof of a chemical interaction between tissue and dye. Griesbach (1888) and Schaffer (1888) acknowledged Paneth's definition of the phenomenon and accepted his terminology.

An indirect contribution by Ehrlich was the interest he stimulated in his students, Pappenheim and Michaelis. In his writings Michaelis recognized his teacher's contributions, but in addition he credited Ehrlich with ideas and terminology which we have not found in the latter's works. For example, Michaelis wrote (1903), "According to Ehrlich (1877) [see above] we mean by metachromasy the phenomenon that a chemically homogeneous dye stains different tissue elements in different colors . . . . With Ehrlich we will designate the dyes as *metachromatic*, the tissue elements *chromotropic*." Yet, in fact, it appears that Michaelis introduced the term chromotrope into the histological literature, as well as the term orthochromatic, both of which are in his book of the previous year (1902 *a*, p. 117). It was after the appearance of this article by Michaelis in Ehrlich's *Encyklopädie der mikroskopischen Technik* (1903) that the concept and terminology became associated with the latter's name.

There were alterations and additions to the terminology after these early papers of Michaelis. To Lehner (1924) the idea (Michaelis, 1903; Hansen, 1908) that a subtly modified dye is selected by the chromotrope violated the metachromasy concept, therefore he suggested as a suitable name, "**allochromasy**."

The term "*echte*" (fast) or "true" metachromasy has been used to distinguish the relatively stable metachromasy of such structures as cartilage and mast cell granules which are not sensitive to alcohol and other reagents (Michaelis, 1902 *a*; Hansen, 1908; W. and M. von Möllendorf, 1924; Lison, 1935 *a*, *b*, 1936 *a*, *b*; Sylvén, 1941). In contrast to "true metachromasy" is "semi-metachromasy" (attributed to the von Möllendorfs by Lison and Mutsaars, 1950), namely, a spurious color change that can be destroyed by various reagents. Lison and Mutsaars (1950) also speak of "positive" and "negative" metachromasy to contrast respectively the typical hypsochromic color shift (spectral absorption shift to shorter wave lengths) with the bathochromic one (to longer wave lengths) that can occur at other times with these dyes.

### *The Meaning of Metachromasy; Relation to the Controversy over the Mechanism of Staining*

Several aspects are important in an analysis of metachromasy: the dye itself, the factors which promote the color change in dye solutions, the substrates which induce this phenomenon, and the interaction between these latter and the dye.

G. Bouma (1883) was the first person to consider in detail the chemical aspects of the color change. Though ignored, his work is outstanding; more than 50 years were to pass before a comparable study would be made (Lison, 1935 *a*, *b*, 1936 *a*). Bouma spectroscopically characterized the red color of the dilute (1:2000) safranine solution which stained cartilage yellow. He also studied the stained cartilage with a microspectroscope. The dye solution became yellow when mixed with chondrin extracted from cartilage. The yellow solution reverted to the original red color upon heating and the effect reversed upon cooling. The red color also was recalled by the addition of small amounts of alcohol or large amounts of acetic acid. Since the absorption spectra of the stained cartilage and the chondrin-safranine solution corresponded, Bouma concluded that the yellow cartilage stain depended upon the combination of safranine with chondrin present in the matrix. Not understood then, lost until now, this work had no effect.

The idea that pure dye can stain tissue components in more than one color immediately had repercussions in the dispute over whether staining is a chemical or physical process, since the color change was interpreted as a sign of intimate chemical association (Paneth, 1888). The proponents of a physical concept of staining led by Fischer (1899) insisted on the presence of more than one dye. Studies were burdened with this question for many years (Ball and Jackson, 1953; Bouma, 1883; Fischer, 1899; Griesbach, 1888; Hoyer, 1890; Kramer and Windrum, 1955; Lehner, 1924; Mann, 1902; Mayer, 1896; Paneth, 1888; Schaffer, 1888; Singer, Weiss, and Dempsey, 1951).

Even prior to Fischer's objections, Hoyer (1890) had decided that, whereas the variable results obtained

with certain dyes were best explained on the basis of contaminants, his thionine was pure and showed color differences depending upon the solvent and, indeed, in aqueous solutions even upon the temperature.

The similarity between the metachromatic color and the color of alkaline dye solutions influenced the thoughts of investigators for many years. Hardy (1891) observed the rose and blue colors of various structures stained with methylene blue and suggested that interaction with the substrate altered the dye chemically, and therefore its color, in a manner reminiscent of the action of alkali. Unna (1891) raised the persistent doubt whether methylene blue is a metachromatic dye—a suspicion which no longer has any justification. Unna obtained redder staining of mast cell granules with aged alkaline solutions. Believing that methylene violet arises under these conditions (Bernthsen, 1885), he attributed the red color to this dye, although he failed to obtain similar results with pure methylene violet in a number of solvents.

Pappenheim (1901) contended that chemical factors more subtle than simple charge relationships are involved in staining (p. 158) because while all basophilic (acid) structures are similarly charged, only a few stain metachromatically and these affect the dye color like alkali treatment. In his monograph Michaelis (1902 *a*) also remarked that (p. 117): "One could now be tempted to interpret metachromasy as if the chromotropic substances reacted strongly alkaline and behaved toward the dye like sodium hydroxide." He postulated a tautomer whose chemical constitution and color resemble the imine base which is liberated by alkali treatment. He assumed for the thiazines, as an example, that an HCl group can migrate from the terminal to the central nitrogen and form a red tautomer. Metachromatic staining then depended upon the selection of the red tautomer by the chromotrope. Methylene blue does not possess the necessary HCl grouping, and so Michaelis classified it as a non-metachromatic dye. He attributed the metachromatic properties of polychrome methylene blue to the presence of the less methylated azures.

Sustained interest in the resemblance between the metachromatic color and the color of alkaline dye solutions led to controversy inasmuch as investigators did not always distinguish between the various bases that may form. Pappenheim pointed out in his monograph (1901, p. 180) that carbinol bases are generally colorless. Yet, Bargum (1903) explained Pappenheim's concept of an alkali-like effect of the substrate in terms of this type of base, and Pappenheim, himself, then affirmed this view (1905, 1906). Similarly, Hansen (1908) believed that aqueous dye solutions contain appreciable amounts of a "hydrolytically formed free base" and that "certain histological constituents... accumulate the previously existing hydrolytically formed free base...." Hansen's "hydrolytically formed, free base" was invoked by Eisenberg (1910) to explain the differential coloring of lipides by aqueous solutions of basic aniline dyes. The free base was supposed to be more soluble in lipide than in water. Pappenheim (1910 *a*, *b*) took issue with Eisenberg for crediting the concept of the free base to Hansen and he exchanged polemics (1911 *b*, *c*) with Scott (1911 *a* to *c*) who exposed the naivete of the carbinol base concept.

The idea of the importance of dye aggregation for metachromasy was introduced by Mann (1902). After pointing out (p. 386) that the position of chromophores has a great influence on color, he stated: "Analogous changes, I believe, may account for some of the metachromatic effects produced in tissue by the use of simple dyes, although in most cases we are dealing with different sized aggregates of the dye."

Now aware of the difficulties inherent in relating metachromasy to the color of alkaline solutions, Michaelis (1910) dropped the tautomer concept and considered two possibilities, that the ion was blue and the undissociated salt red, or that the red color belonged to a polymer. The first possibility was rejected because alcoholic solutions are blue instead of red. The second idea became plausible when molecular weight determinations in aqueous solutions yielded higher values than were expected. The color-controlling factor now became the equilibrium constant for a given medium.

During the next two decades, colloidal aspects were brought progressively into focus on the problem of metachromasy. Ostwald demonstrated (1919) that changes in degree of dye dispersion are responsible for the color changes which follow alteration in the concentration of Congo rubin. It was, however, the von Möllendorfs (1924) who brought colloidal aspects of staining to the attention of histologists. They related the intensity of staining of ligamentum nuchae to the colloidal nature of dyes. The colloidal character was assayed by the rate of penetration into gelatin. They also noted that basic dyes are precipitated by acid colloids and suspensions of yeast nucleic acid, and that the precipitate is metachromatic with the so called metachromatic dyes. They grouped the staining behavior of dyes into two morphological classes: "*Durchtrankungsfärbung*" (penetration staining) and "*Niederschlagsfärbung*" (precipitation staining). The former type was common to both acid and basic dyes, whereas precipitation at surfaces was characteristic of basic dyes. Since basic dyes are almost exclusively involved in color changes on staining, metachromasy became a specialized expression of the precipitation of basic dye at surfaces.

In 1926 and 1927 Pischinger published his work on the physical chemistry of staining; the results attacked the foundations of the von Möllendorf concept. Following experiments with isolated proteins and tissue sections in buffered solutions, he concluded that staining with acid or basic dyes depends upon the nature and the extent of the charge on the protein. These in turn are a function of the isoelectric point of the protein and

of the solution environment, namely the pH. According to Pischinger, staining has to be considered from the common point of view of tissue charges and change in tissue charges. He was inclined to regard metachromasy as the formation of a compound having an intrinsically different color.

Czaja (1930, 1933) attempted to combine aspects of the colloidal and free base concepts in a study of plant materials. He attributed metachromasy to an ionized form of the free base which was assumed to form by an alkali "*Membranporen*" effect. He supposed that a surface with an adsorbed layer rich in hydroxyl ions dissociated anions and that these passed into the external ion atmosphere to elevate the pH. Thus a basophilic substance would have an alkaline effect upon dye cations.

A line of research more closely allied to Bouma's approach appeared in the work of Holmes (1924), who studied the effect of dye concentration on the absorption spectra of aqueous solutions. He noted the progressive appearance of a secondary band at shorter wave lengths and the concurrent disappearance of the primary band at the longer wave lengths. Holmes postulated a new tautomeric explanation of metachromasy (1926, 1927), namely, that a change in the valence of nitrogen from 3 to 5 might be responsible for the color change. Meanwhile, Michaelis (1926), who had abandoned the tautomer concept of metachromasy (1903) and had suggested dispersion effects (1910), raised some doubts about the dispersion theory.

The impetus for modern histochemical interest in metachromasy was supplied by Lison (1933; 1934; 1935 *a*, *b*; 1936 *a*). Lison ascribed histochemical significance to the coloration when it appears or persists under specified conditions. He developed Michaelis' (1902 *a*, 1903, 1910, 1926) classification and extended the list of known metachromatic dyes. Lison, unlike many of his predecessors, carefully distinguished the imine, carbinol, and ammonium bases of dyes and decided that while all metachromatic dyes were capable of forming imine bases, the reverse was not necessarily true. The former property excluded methylene blue. Lison reaffirmed the fact that metachromasy depends upon concentration and temperature and that the phenomenon is reversible. He unknowingly repeated Bouma's (1883) observations and reported that the metachromatic color develops in very dilute solutions in the presence of minute amounts of chromotropic substances and that the color under such circumstances is also sensitive to temperature and alcohol. He concluded that the phenomenon is essentially the same in the presence or absence of the chromotrope and that various chemical and physical factors merely displace an equilibrium which ordinarily exists in aqueous solution between the "normal" and the "metachromatic" form of the dye. He emphasized that the metachromatic absorption shift is invariably hypsochromic (to shorter wave lengths) and that water is essential. He rejected the polymer concept because as a rule the color change due to colloidal aggregation is bathochromic (to longer wave lengths). Pischinger's notion of intrinsic color change was rejected because of the reversibility of metachromasy. Lison also rejected (correctly) the tautomer concept since his curves (incorrectly) showed general displacement rather than a restricted shift in the spectrum. In his words: "The nature and constitution of the metachromatic form are not entirely defined; it is certain that it is not the free imine base of the dye; probably it is neither a tautomer nor a purely physicochemical modification of the normal form. Perhaps it could result from an intramolecular transformation of the dye accompanied by a reduction of its dispersion." He concluded on the basis of elaborate histochemical studies (see below) that under well defined conditions the color change is a specific test for the sulfate esters of polysaccharides of high molecular weight.

The views of Kelley and Miller (1935 *a*, *b*) contrast sharply with those of Lison. These authors regarded methylene blue as an excellent metachromatic dye. Like Holmes (1924), they observed two peaks. When the dye was bound to mucin or when it was concentrated in aqueous solution the major peak was depressed and the minor peak became more prominent and was displaced toward even shorter wave lengths. Nucleoprotein or nucleic acid, on the other hand, caused the color to shift to the higher wave lengths—an effect reminiscent of that of alcohol. Kelley and Miller rejected the tautomer concept as too restricted. They discarded as well the idea that metachromatic staining depends upon selective interaction of the substrate with some activated form because their data seemed to show that the form of the dye is determined after union. Further evidence was the fact that mucin when stained in alcoholic solution is blue but immediately becomes violet when the alcohol is replaced by water. They also rejected the thought that concentration on the substrate is responsible for metachromasy because mucin, even when weakly stained, was violet while intensely stained nucleoprotein was blue. They favored the theory "that a change in the angle of the valency bonds of the nitrogen" is responsible. They developed the concept in terms of J. Moir's (1927) color theory, namely that when the substrate stains metachromatically the dye is bound in a "contracted form."

The idea that dye aggregation is a prerequisite for the shift continued in favor. Scheibe (1937, 1938) established that the color changes in concentrated aqueous solutions are dependent upon the formation of micelles. Lison and Fautrez (1939) decided that while "the nature of the metachromatic form is not established with certainty, it appears that it results from the polymerization and tautomerization of the dye salt." Developing the aspect of colloidal aggregation, Bank and Bungenburg de Jong (1939) took issue with Lison's characterization of metachromasy and said, "We can characterize metachromasy spectroscopically as the

excitation of a new 550 mμ band and the disappearance or respective weakening of the two bands characteristic of the dilute aqueous toluidine blue solution." They emphasized that the color intensity is minimal at maximal metachromasy. Their study of sulfate, phosphate, and carboxyl colloids (see below) led to the concept that ". . . metachromasy is to be interpreted as the consequence of the electroadsorption of the dye cation to the ionogenic sites of the negative biocolloid." The effect of alcohol on metachromasy when related to length of the chain led them to the conclusion that van der Waal's forces are involved.

They believed that metachromasy in dye solution or in stained tissue is due to dye aggregation. A dye solution would become metachromatic as dye concentration increased and as temperature decreased. Strongly hydrated ions would decrease the available solution water and induce aggregation. By virtue of its carbon skeleton, alcohol could penetrate the aggregates and then with its polar hydroxyl groups weaken the aggregative forces. The concept also accorded with the metachromatic coloration of powdered toluidine blue.

Spek (1940) concluded that these dyes precipitate and form dehydrated complexes with chromotropic agents. He thought that the metachromatic color is a property of the undissociated dye salt.

Rabinowitch and Epstein (1941) interpreted their quantitative studies of the behavior of methylene blue and thionine in aqueous and alcoholic solution as consistent with the dimer hypothesis and aggregation under the influence of van der Waal's forces supplemented by Heitler-London forces. Assuming these forces, they then calculated the equilibrium distance as 3.12 A.

Sheppard and Geddes (1944) recognized difficulties in the dimer hypothesis, but felt it nevertheless explained the dilution effects best. They pointed out that the dimer absorption band is not new but arises at the site of a "bump" which is present in extremely dilute solutions. The bump was attributed, in quasi-classical terminology (Lewis and Calvin, 1939), to the partial resolution of the vibrational structure of a single electronic band (Lewis *et al.*, 1942; 1943 *a* to *c*). They reemphasized the necessity of water which according to them acts as an electronic buffer between the charged ions and permits aggregation by van der Waal's forces.

The polymer concept was reemphasized by Michaelis (1944) who now ascribed metachromatic staining to the adsorption of a polymolecular layer held together by exaggerated van der Waal's forces (1945, 1947). He, too, was converted to using methylene blue because its metachromasy is spectroscopically well defined. The associated decrease in color intensity was explained by the assumption that ion clusters absorb less light than homogeneously distributed ions. The source of the absorption bands was, however, less certain, and he noted (1947), "All we can say is that the energy of quantitation of the molecule is not the same in the monomer as in the di- or polymer, or that the probabilities of transition from one energy level to another are altered on polymerization." Whereas he had previously distinguished three specific bands, the monomer ($\alpha$), the dimer ($\beta$), and the polymer ($\gamma$), he now concluded that there was not a specific $\gamma$ band but rather a series of progressively overlapping bands due to a progressive increase in the size of the polymer. Finally, he accepted as probable Sheppard's concept of the nature of the bands and the role of water in the development of metachromasy.

Wiame (1947) and Bungenberg de Jong (1949) related the degree of metachromasy with the number of negative sites. Wiame conceived of the dye as "polymerized" through the substrate, a view essentially like that of Bank and Bungenberg de Jong (1939). However, Lison and Mutsaars (1950) with non-chromotropic nucleic acids obtained maximal metachromasy when the dye to phosphate ratio was 500 rather than unity. Study of the color changes of nucleic acids under various conditions of staining led Lison to abandon his earlier view that a hypsochromic shift is characteristic of metachromasy. He found that very lightly stained nuclei are distinctly green (bathochromic change) rather than the "normal" blue color of the dye. Because this effect is also abolished by heating, the term "negative metachromasy" was introduced to distinguish it from the usual "positive metachromasy."

In his last publication (1950), Michaelis questioned his previous view that chromotrope metachromasy is due to binding of the dye as a polymer because ". . . the interpretation of the $\gamma$ band as that of a high reversible polymer of the dye has become doubtful as to its generality . . . . When a 1 to 3 per cent agar gel is stained . . . at such a low concentration of the dye that the color is distinct only upon looking through the whole length of the test tube, the color is still at room temperature very decidedly the pink, metachromatic color . . . it is unlikely that the dye should have been adsorbed in the form of polymolecular dye micelles, rather is a monomolecular distribution of the dye over the negatively charged sites of the colloid the only reasonable assumption."

Anticipating the present report, we indicated in a review (Singer, 1954) that "it seems reasonable to suggest as an alternative hypothesis that molecular binding occurs instead but that the binding sites of chromotropic substances are clustered and so arranged in pattern and density that adjacently bound dye ions are close enough to interact optically even when bound in low concentration." In an extensive report on the interaction of the dye with a large number of substrates, Sylvén (1954) also expounded the concept that metachromasy occurs upon orderly binding of dye to serially repeated anionic sites. To explain the color alteration he invoked the formation of new intermolecular bonds which have an energy requirement of about 8 calories and proposed a minimum intercharge distance of approximately 5 A. According to him close spacing

increases metachromasy. He emphasized the necessity of water and pointed out that dye alignment is favored if the molecules present hydrophobic and hydrophilic parts.

A tentative operational definition of metachromasy may be offered based on information gathered *to this point*: Metachromasy is the hypsochromic (shift in absorption to shorter wave lengths) and hypochromic (decrease in intensity of color) change in color exhibited by certain basic aniline dyes in the presence of water and under the following conditions:

1. Increase in dye concentration.
2. Temperature decrease.
3. Salting out.
4. Interaction with certain substrates whose metachromatic influence may be due to serially arranged proximate anionic sites.

*Histochemical Significance of Metachromasy*

The modern histochemical applications of metachromasy are described in various recent treatises (Lillie, 1954; Pearse, 1953; Lison, 1953; Gomori, 1952; Carnes and Forker, 1956; Kelly, 1956; Schubert and Hamerman, 1956), and certain aspects need only be touched upon here.

It is noteworthy that from the very beginning (Beneke, 1862) specific reagents were sought among the synthetic dyes. It was soon found that *amyloid* (Baumgarten, 1876; Cornil 1875 *a* to *d*; Heschl, 1875; Jürgens, 1875; etc.), *cartilage* (Cornil, 1875 *a* to *d*; Ranvier, 1875; etc.), *mast cell granules* (Ehrlich, 1877, 1878, 1879 *a*, *b*; etc.), *mucus* (Ehrlich, 1877; Podwyssotzky, 1878; Schiefferdecker, 1878; etc.), *myelin* (Adamkiewicz, 1882, 1884 *a*, *b*; Treitel, 1876; Carl, 1877; etc.), and *nervenkörperchen* (Adamkiewicz, 1885 *a*, *b*; 1890; etc.) stained in a different color from that of the staining solution.

Bouma (1883) characterized the phenomenon (see above) and showed that safranine reacted with chondrin extracted from cartilage to yield a heat, alcohol, and acid labile compound of altered color that was spectroscopically identical with the yellow color produced *in situ* when cartilage was stained with this red dye. Unfortunately, this work had no effect on later studies; contemporary editors and abstractors saw in it only a recommendation of safranine as a cartilage stain.

Another early example worth noting of the use of metachromasy in the analysis of tissues is the study of Reich (1903) who reexamined Adamkiewicz's "Nervenkörperchen" (see above). Reich concluded that the properties of the chromotrope coincided with those of protagon, a phosphorus-containing lipide prepared from brain by Liebreich (1895).

The modern purposive use of metachromasy in histochemistry stems mainly from the generalizations of Lison which were forcefully presented in his treatise of histochemistry (1936 *b*). He found that as little as $5 \times 10^{-4}$ mg. of chondroitin sulfate could be detected by metachromasy. Similar results were obtained with mucoitin sulfate, and two new chromotropic sulfate esters, agar and carrageen. Lison showed in various ways that metachromasy is characteristic of high molecular weight sulfate esters. A series of simple sugars and lipides were non-metachromatic, as was another series of polysaccharides—gums, mucilages, cellulose, glycogen, etc. Nitric, phosphoric, and silicic esters of high molecular weight were also non-metachromatic. When converted to sulfate esters, many of these substances now became metachromatic, provided that the molecular weight was high. Lison concluded: "The metachromatic *virage* of a dye is a true chemical and histochemical reaction; perfectly reproducible and rigorously specific for a group of well defined chemical substances, it is the exclusive property of sulfate esters of quite high molecular weight, among these esters the biologically more important are those derived from polysaccharides. . . . Everything that stains by a metachromatic dye like toluidine blue, in a shade different from the normal (blue) color is not a chromotropic element. In order to be able to consider it as such, it is necessary that it stain in a color different from the normal form, *under conditions where the latter is normally the only stable one.* These conditions are fulfilled under the following circumstances: in an aqueous solution when the concentration at the level of the tissue is weak, that is to say the coloration is light, or the pH is low (in practice this condition can be regarded as fulfilled when the pH is less than 3), or when the temperature is high (rather impractical); or, in non-aqueous media when the coloration persists in glycerin, levulose syrup, Apathy's gum syrup, and *a forteriori* in Canada balsam. It is under these conditions that metachromasy attains histochemical significance; otherwise, it is only a commonplace manifestation, like the color changes in pure aqueous solution and has absolutely no diagnostic value."

Sylvén (1941) suggested a faster technique for determining "*echte metachromasie*," namely the use of more concentrated but alcoholic dye solutions followed by dehydration for an arbitrary time in alcohol. At approximately the same time, a number of investigators (Benazzi-Lentati, 1940 *a*, *b*; Bignardi, 1940 *a*, *b*; Clara, 1940; Francini, 1940) reported that normally non-chromotropic substances (mucoid secretion, glycogen, starch) could stain metachromatically after pretreatment with chromic acid, periodic acid, and other substances. This effect had been noted earlier (E. Unna, 1918). Bignardi demonstrated that this effect was due to oxidation, and attributed the metachromasy to newly formed acid groupings.

Bank and Bungenberg de Jong (1939) evaluated the metachromasy of dye interaction with colloids having

sulfate, phosphate, or carboxyl groups. Within each ionogenic group the members were arranged in order of increasing charge density. In each case an increase in charge density could be correlated with an increase in metachromasy. Moreover, metachromasy increased in the following order of the three groups: carboxyl, phosphate, sulfate.

As they expected, pretreatment with certain inorganic ions also induced metachromasy. This was particularly true of the complex polyvalent anions, phosphomolybdic and phosphotungstic acids. Various cations and anions decreased the metachromasy of a maximally metachromatic mixture of toluidine blue and arabinate. The effectiveness of the cations increased with their valence except in the case of hydrogen ion, which was more effective than the divalent cations of the alkaline earth series. Recent examples of the metachromasy of inorganic substances have been presented by Wiame (1947) for hexametaphosphate and by Merrill and Spencer (1948) for silicates.

Michaelis (1947) showed that various adsorbents influence the absorption spectrum of concentrated metachromatic solutions of toluidine blue. When large amounts of sodium oleate and aerosols 18 and 22 were added, the $\beta$ band disappeared and only a prominent $\alpha$ band remained. As the relative amount of dye was increased the $\beta$ band reappeared and finally a $\gamma$ band as well. Chromotropes were stained metachromatically for all values of the ratio, dye: adsorbent. Solutions of nucleic acid never developed a $\gamma$ band and, therefore, this substance he classified as unique.

Lison and Mutsaars (1950) studied dye interaction with nucleic acids and maintained that metachromasy both "negative" and "positive" develop under the appropriate staining conditions. Mixed solutions of dye and nucleic acid were green when the dye:nucleic acid ratio was low, and the same color occurred with lightly stained nuclei. Since heating of the solution reversed the color to the orthochromatic blue, they designated this phenomenon "negative metachromasy" to distinguish it from "positive metachromasy" (hypsochromic color shift). As noted already, this view altered Lison's earlier insistence (1935 *b*; 1936 *b*) upon the essentially hypsochromic nature of the color shift. They applied the term "semi-metachromatic," ascribed to von Möllendorf (1924), to substances like nucleic acid which show "positive metachromasy" only under certain optimal conditions to distinguish them from other substances, like the sulfate esters, which develop it readily.

Landsmeer (1951) used the effect of cations on metachromasy as a histochemical tool to determine whether staining with a basic dye is due to carboxyl, phosphate, or sulfate groups. Flax and Himes (1952) studied the difference between the two nucleic acid types and decided that RNA stains more metachromatically than DNA. This distinction was attributed to differences in the density of available phosphate groups.

In his recent study, Sylvén (1954) has also reported that given the same periodicity of anionic sites, metachromasy increases in the order carboxyl, phosphate, sulphate. Further, the carboxyls weaken in their effect on metachromasy as the chain length increases.

In summary, recent studies reflect the fact that the forces and groups which bind the dye to the substrate have become of increasing importance in the evaluation of metachromatic staining. The availability of the binding sites is a direct function of the solution environment, notably pH and ionic strength (Singer, 1952). The sites must be suitably spaced. Moreover, the degree to which the sites are saturated appears to determine the extent of the color shift. It has also been indicated repeatedly that the sequence of carboxyl, phosphate, and sulphate are of increasing importance for metachromasy in that order, but this arrangement may, in part at least, be an expression of the disposition and availability of the sites under the specific staining conditions employed. Lastly, there is the unexplained necessity for water (see discussion).

## EXPERIMENTAL

### *Materials and Methods*

Fibrin film was prepared from human fibrinogen and thrombin by the procedures of Ferry and Morrison (1946, 1947). The films, previously dried in air at room temperature, were fixed for 6 hours by immersion in 10 per cent neutral formalin, washed overnight in running tap water, dried in air at room temperature, and stored in a refrigerator. The dry film contained about 2 mg. of fibrin/cm$^2$. The thickness of the fixed dry film—measured in optical section with a revolving barrel type ocular micrometer—was approximately 30 microns. The thickness can be regarded as constant during subsequent manipulations (Ferry and Morrison, 1947).

In most experiments, pieces of film were stained in buffered solutions of methylene blue, a dye whose metachromasy is very well defined spectrophotometrically (see below). Certified methylene blue (National Aniline 30, 85 per cent dye content) was employed. Its spectral absorption did not differ appreciably from a sample repurified by the method of Holmes (1928). In fact, the original sample was relatively free of the less methylated homologues. The spectral absorption curves of varying concentrations of the sample at constant pH (6.0) and ionic strength (0.02) are shown in Fig. 1.

The film was stained to equilibrium in dilute (non-

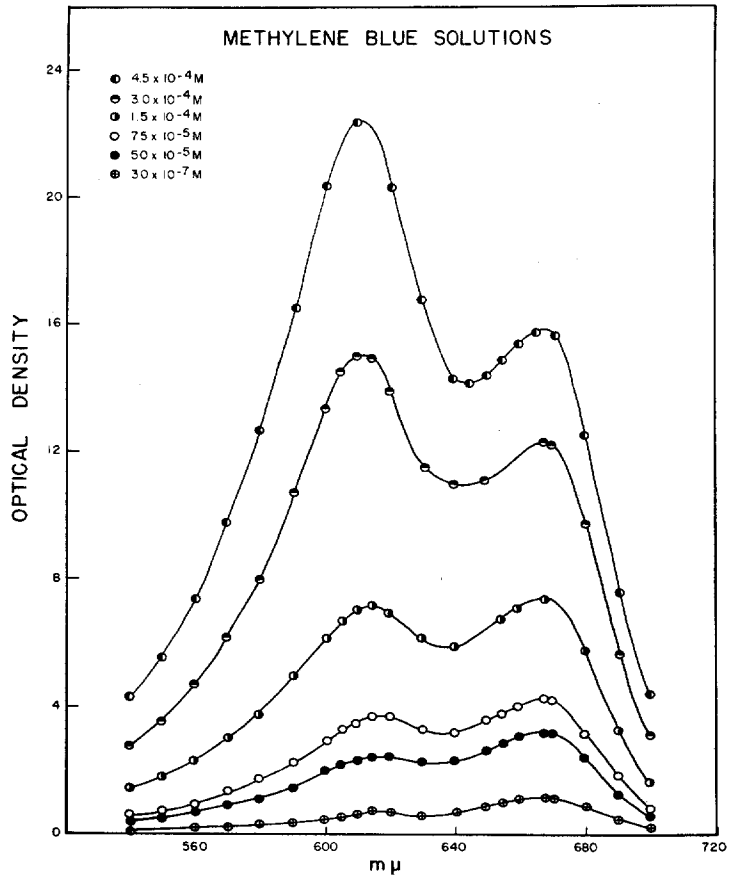



FIG. 1. The "dilution shift" of the absorption spectra of methylene blue in veronal-HCl buffer of low concentration ($S \cong .02$) pH 6.0 and at 20°C.

metachromatic) dye solutions ($3 \times 10^{-7}$ M to $5 \times 10^{-5}$ M) by immersing a rectangular piece (5 to 8 cm.$^2$) in 100 ml. of staining solution for 24 hours. The solution was agitated slowly and maintained at constant temperature (25°C.). Solutions were buffered between pH 6 and 9 with Michaelis HCl-sodium veronal buffer at constant ionic strength (0.02).

The absorption of stained film was determined with a spectrophotometer (Beckman, model DU) by comparison with samples of the same films which had undergone similar treatment but in the absence of dye. The wet films were mounted between glass slides which, in turn, were secured in a special holder. Caution was exercised to exclude air bubbles. The boundary of the film was traced and the area determined planimetrically; then the bound dye was extracted from the film in a small volume of 0.001 N HCl. The extract was diluted to a known volume with a solution buffered at pH 6 and of an ionic strength of 0.02; and the dye concentration was measured spectrophotometrically. From the measurements of dye concentration and of film area and thickness, the bound dye concentration could be expressed as molarity.

In another experiment, small pieces of fibrin film were embedded in 25 per cent gelatin. The chilled gelatin blocks were fixed in 10 per cent neutral formalin in the refrigerator for 24 hours. The blocks were washed overnight in running tap water and then sectioned at 10 microns with the freezing microtome. The sections were affixed to slides by drying. These sections and intact films were stained in 0.1 per cent toluidine blue in 30 per cent ethanol as suggested by Sylvén (1941); but, in addition, the pH of the dye bath was controlled between 5 and 9. The preparations were treated in several ways. After staining, some specimens were mounted in aqueous media buffered to the staining pH. Other samples were dehydrated in absolute ethanol (10 minutes–2 days) or by heating at 120°C. (1 to 24 hours); they were then cleared and mounted in clarite or rehydrated and mounted in the buffered aqueous medium.

The tracheae of several mice (strain C57) were fixed

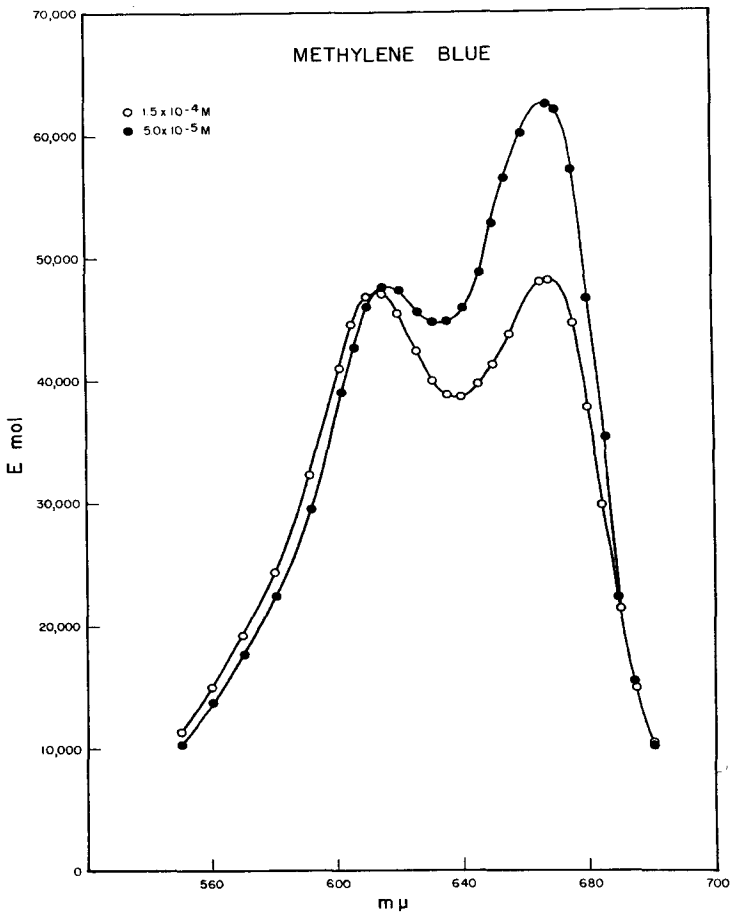



FIG. 2. The hypochromic character of metachromasy appears when absorption spectra are compared on a molar basis (from Fig. 1).

in 10 per cent neutral formalin or in Helly's fluid, embedded in paraffin, and sectioned at 5 microns. Each slide carried sections from tissue fixed both ways. The preparations were stained to equilibrium (24 hours) in large volumes (1500 ml.) of slowly agitated toluidine blue solutions of constant dilute concentration ($10^{-5}$ M) and low ionic strength (0.02) but of varying pH (2.5–8.7). The preparations were mounted in equilibrium fluid under paraffin-sealed coverslips. After examination the coverslip was removed, excess fluid was blotted, and the samples dried at first in an evacuated desiccator containing drierite (24 hours at 60°C.) and then in a drying oven (110°C., for 24 hours). They were then cooled over drierite, flooded with anhydrous xylene, and mounted in harleco synthetic mounting medium for examination and photomicrography. The color of the stained specimen was observed visually by Köhler illumination supplied by a research lamp (Bausch and Lomb) equipped with a ribbon filament source and a daylight filter (Corning). To extend the comparison of the color of hydrated and dehydrated specimens, some sections were rehydrated after removal of the coverslip and extraction of the medium in xylene.

The role of water in the development of metachromasy in chromotropic substrates was also studied in films of 0.5 per cent agar (Difco) in 10 per cent gelatin, 0.5 per cent heparin (Premo Pharmaceutical Laboratories) in 10 per cent gelatin, and 3 per cent agar. To prepare the film, warm solutions containing a trace of wetting agent (2 drops/100 ml. of A and E aerosol) were pipetted into the shallow recess (approximately 7.4 cm.$^2$) of inverted glass covers of staining dishes. After solidifying in the cold, the gels were denatured with cold formalin vapor for 12 to 24 hours. The fixed films were then stripped, washed for 1 hour in dilute buffer, and stained in solutions of methylene blue or toluidine blue ($10^{-5}$ M) containing dilute (1/10) McIlvaine buffer (pH 3.5, 20°C.; staining time—24 hours in 1500 ml. of agitated solution). The

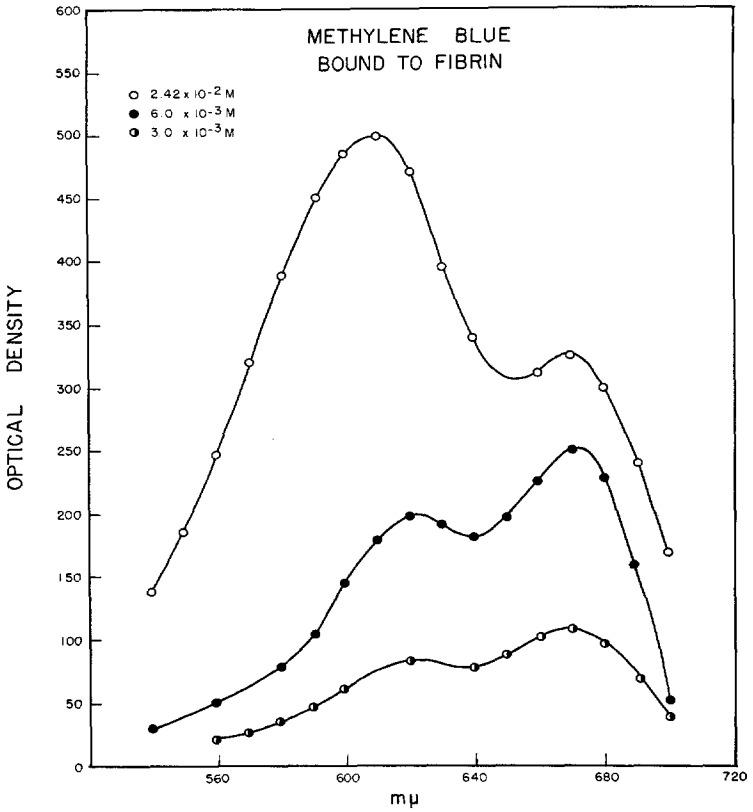



FIG. 3. The spectral absorption of methylene blue bound to fibrin film. There is an initial suppression and a delayed expression of metachromasy by comparison with aqueous solutions (Fig. 1).

control film was treated in similar solutions having no dye. The stained films were examined spectrophotometrically (Beckman Model H) in the equilibrium fluid against control films in the dilute buffer. The films were reexamined at intervals after varying degrees of dehydration, and again after rehydration. Progressive dehydration was achieved by the following procedures which were used singly or in various combinations:

1. Desiccation over drierite, 20°C., 12 to 24 hours, *in vacuo*.
2. Desiccation over drierite, 60°C., 12 hours–3 days, *in vacuo*.
3. Desiccation over $P_2O_5$, 20°C., 12 hours–1 week, *in vacuo*.
4. Oven desiccation, 110°C., 12 to 72 hours.
5. Oven desiccation, 140°C., 6 to 12 hours.

Rehydration was performed either slowly by exposure to a water-saturated atmosphere or rapidly by immersion in distilled water.

## RESULTS

The absorption of varying concentrations of our sample of methylene blue in dilute (S = .02) veronal-HCl buffer of pH 6 at 20°C. is illustrated in Figs. 1 and 2. Two maxima are visible, the higher wave length, $\alpha$ band and the lower wave length, $\beta$ band. The $\alpha$ band is stable in position at 667.5 m$\mu$, but the $\beta$ band shifts from approximately 615 m$\mu$ to 610 m$\mu$ with increase in dye concentration. As the dye concentration is elevated metachromasy develops; the $\beta$ band (Fig. 1) becomes more prominent than the $\alpha$ band and shifts hypsochromically. When absorption is expressed per mole of dye, it becomes apparent that at higher concentrations both peaks are actually reduced but the $\beta$ peak less so.

The absorption spectrum of methylene blue bound to films of fibrin was compared to that of dye in solution. The films were stained to equilibrium at specified conditions of dye concentration, pH, ionic strength and temperature. The selected conditions were optional for basic dye binding; the pH was high (8.7) but within the stability range of methylene blue (Holmes, 1929; Rabinowitch and Epstein, 1941), the ionic strength

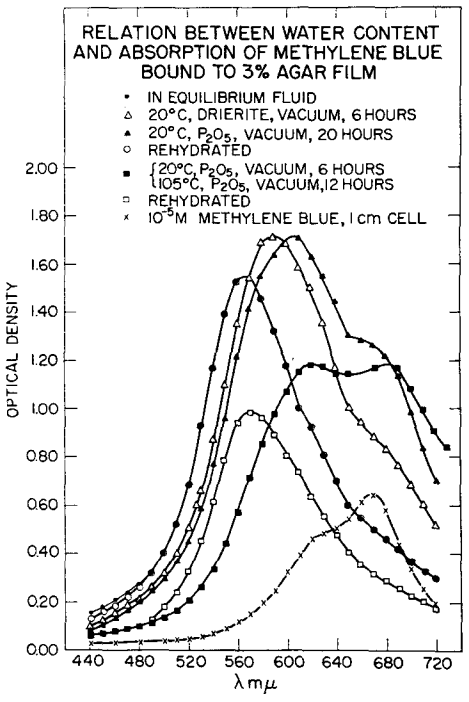



FIG. 4. The film was stained to equilibirum in $10^{-5}$ M methylene blue in dilute (S ≅ .02) McIlvaine buffer of pH 3.5 at 20°C. The phenomenon is perfectly reversible unless some dye has been destroyed at high temperature or extracted by the solvent.

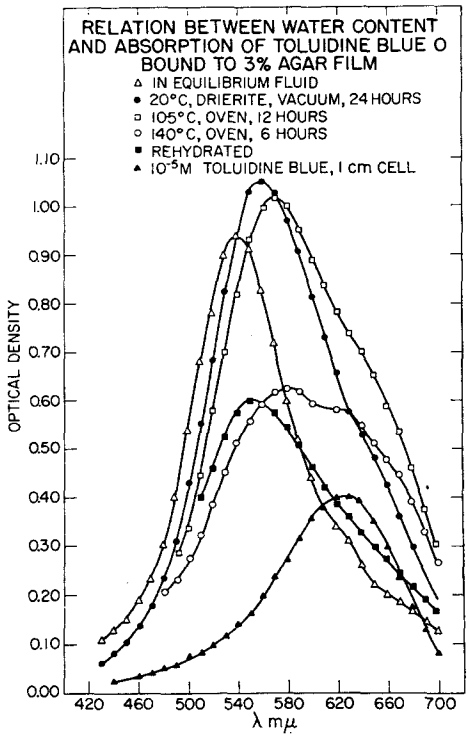



FIG. 5. The film was stained to equilibrium in $10^{-5}$ M toluidine blue in dilute (S ≅ .02) McIlvaine buffer of pH 3.5 at 20°C. Some dye was destroyed at the higher temperatures.

of the bath was low (0.02), and the film was stained to equilibrium (24 hours). Small volumes were used so that the staining bath was practically exhausted of dye. After spectrophotometric examination of the film, the bound dye was extracted in $10^{-3}$ M HCl and diluted in buffer at pH 6. The dye content of the film was expressed as molar concentration from the calculated film volume and the measured extracted dye. Typical results are illustrated in Fig. 3.

The absorption spectrum of bound methylene blue differs in certain important respects from the absorption of the dye in aqueous solutions. The dye concentration in the films represented by the two lower curves exceeds by at least tenfold the highest solution concentration shown in Fig. 1, yet the films are orthochromatic and the solution metachromatic. This is true even if the staining is performed in metachromatic solutions at lower pH (*i.e.*, the same degree of staining may be obtained by elevating the solution concentration but dropping the pH). In addition to such suppression of the metachromatic tendency of the dye, both peaks are shifted slightly toward higher wave lengths, the $\alpha$ peak is at 670 m$\mu$ and the $\beta$ peak is at 620 m$\mu$.

However, as still more dye is bound to fibrin, metachromasy gradually develops. The $\beta$ peak becomes prominent and shifts to 610 m$\mu$, as shown in the upper curve of Fig. 3. The curve is quite comparable in shape and in relative height of the two bands to the upper curve of Fig. 1, although the dye concentration is increased about 100-fold. Fibrin film may be stained even more metachromatically, but then the light absorption is too great for spectrophotometric measurements.

Similar results were obtained with toluidine blue. Frozen sections of the film were cut at 10 microns after embedding in 20 per cent gelatin and fixing in 10 per cent neutral formalin; they were then stained in 0.1 per cent toluidine blue in 30 per cent ethanol at controlled pH. Both fibrin and gelatin were orthochromatic when lightly stained (pH 6.5). At pH 7.5 where much more dye is bound a purple cast developed, and at pH 8 the sections were clearly metachromatic. When the latter

preparations were dehydrated in absolute alcohol or in an oven (120°C.) the metachromasy of the gelatin disappeared. Metachromasy returned immediately upon rehydration. Indeed, the pink color could be recovered by simply breathing upon the preparations. The metachromasy of fibrin, unlike gelatin, was not abolished by dehydration in absolute alcohol (24 hours) or by more prolonged desiccation (36 hours) at 120°C., although the latter did diminish it appreciably. Prolonged exposure to absolute alcohol (3 days) eventually did abolish the metachromasy, but only after quantities of dye had been extracted from the section.

Sections of trachea and surrounding tissues that were fixed in 10 per cent formalin or in Helly's fluid and stained in solutions of toluidine blue at various pH were examined in the equilibrium fluid after desiccation and again after rehydration. The results show that those tissue elements which stain metachromatically lose their metachromasy when dehydrated; and then become metachromatic again when rehydrated.

The metachromasy of individual tissue structures varied with the pH of staining. Mast cell granules and cartilage matrix were metachromatic at low pH (pH 3) where their anionic sites alone were available to the dye. At progressively higher pH levels new anionic sites became available in these structures and staining was more intense, but the metachromasy was progressively masked by the orthochromatic binding of these weaker sites. Conversely, the non-chromotropic elements, first obviously orthochromatic, became increasingly metachromatic as more dye was bound at higher pH.

After formalin fixation, all basophilic components developed some degree of metachromasy. At pH 7 or above, the entire preparation, with the notable exception of the red blood corpuscles, was metachromatic. The most pronounced non-chromotropic metachromasy was evoked in the cytoplasm of the thyroid follicular cells, in the basal cytoplasm of the serous cells of tracheal glands, and in nuclei in general. The metachromasy of these structures appeared first at pH 4. On the other hand, after Helly-fixation, thyroid colloid, nuclei, fibrils of smooth and skeletal muscle, and other ordinarily non-chromotropic structures did not stain as intensely nor did they ever develop an appreciable degree of metachromasy under the conditions of staining employed here.

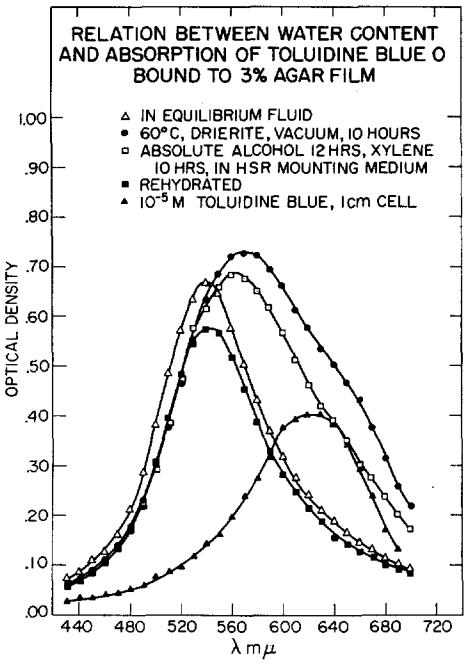



FIG. 6. The film stained to equilibrium in $10^{-5}$ M toluidine blue in dilute (S ≅ .02) McIlvaine buffer of pH 3.5 at 20°C. Some dye was lost in the solvents.

Spectrophotometric study of the dependence of the metachromasy of chromotropes upon water was performed on films of agar in gelatin or heparin in gelatin or of agar alone. The films were fixed in formalin vapor and stained in solutions of toluidine blue or methylene blue. The stained films were examined in the equilibrium fluid in buffer, then after desiccation, and again after rehydration.

Judged visually, even the least effective desiccation procedure (20°C. over drierite) tended to abolish metachromasy. For example, the faint pink metachromatic color of films stained with toluidine blue changed to an intense deep blue.

The absorption spectra (Figs. 4 to 6) show that desiccation at room temperature caused: (*a*) progressive return of the $\beta$ peak to the position occupied in dilute solutions; (*b*) progressive elevation of the $\alpha$ band; (*c*) increase in total absorption which was proportionately greater for the $\alpha$ band.

Rehydration at this point caused a qualitative and quantitative return to the original metachromatic spectrum (Fig. 4). Limited desiccation does not completely reverse the metachromasy as judged by the comparison of the spectra of such dried specimens (Figs. 4 to 6) with those of dye

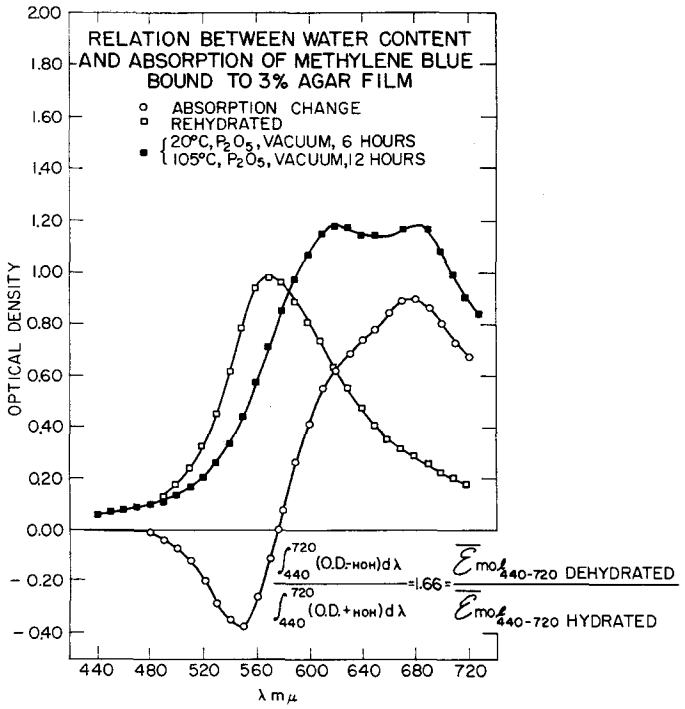



FIG. 7. Data from Fig. 4. The difference between the two spectra is plotted to show the absorption change due to hydration. In the range 440 to 720 mμ, the absorption on a molar basis is 1.66 times greater in the absence of water.

in alcoholic or dilute aqueous solution. The degree of dryness necessary for the elevation of the $\alpha$ band above the $\beta$ band was achieved by desiccation at elevated temperature (Fig. 4). However, the total absorption decreased reflecting the loss of some dye in the process. The loss did not prevent the return of metachromasy upon rehydration, but the total intensity was proportionately reduced here too, and the degree of metachromasy somewhat depressed. The qualitative and quantitative aspects of this reversible color change are visualized by a plot (Fig. 7) of the net change in absorption in this spectral range. In the chosen example the total absorption between 470 and 720 mμ in the dehydrated state is 1.66 times as large as the absorption when water is present.

## DISCUSSION

### *The Suppression and Delayed Expression of Metachromasy by "Non-Chromotropes":*

The studies of methylene blue and toluidine blue bound to fibrin and to sections of tracheal and associated tissue lead to the concept that the so called "non-chromotropes" (*i.e.* non-metachromatic substances) suppress the normal metachromatic tendency of these dyes. Fibrin films are orthochromatic with internal dye concentrations that far exceed the level at which metachromasy develops in aqueous dye solutions; and most cellular and tissue structures including the nucleus may also be stained quite deeply and yet be orthochromatic. Nevertheless, a visible and measurable metachromasy eventually does develop in fibrin films if still more dye is bound. Likewise most non-chromotropic elements present in tissue sections become progressively metachromatic as more sites are recruited, for example at successively elevated pH levels of staining (see Results).

Given our present understanding of metachromasy and of the factors that control dye binding, it seems reasonable to explain the suppression of metachromasy not on the chemical nature of the binding site, but rather upon the distribution and frequency of the dye-binding sites, an extension of ideas which originated with Bank and Bungenburg de Jong (1939). A direct influence of the binding site may be reflected in the very slight shift toward higher wave lengths initially shown by both peaks. As dye is bound it tends to distribute itself

throughout the system and not to satisfy only local or surface sites (the socalled "levelling" effect of textile colorists). In non-metachromatic systems the dye cations are distributed over sites which are too far removed for interaction, a prerequisite for metachromasy (see below). Yet, the same dye concentration in aqueous solution may be metachromatic because the cations are free to aggregate under the influence of various forces, whereas in the stained substrate the coulombic forces involved in levelling and binding preclude such interaction. As more dye is bound in such a system, the chance increases that some dye ions will be bound within the range of mutual influence. Sylvén (1954) has estimated that the binding sites cannot be separated by more than 5 A. Thus even in non-chromotropic systems metachromasy can be expected and, as we have seen, occurs when a saturation of available anionic sites is approached.

Therefore, the idea is advanced that some degree of metachromasy can be evoked in any system under aqueous conditions provided that binding sites can be recruited at suitably close intervals for interaction of bound dye ions. The extent of the shift of absorption in such a system is a function of the average frequency of such interactions in the population of bound dye. If the frequency is low, its effect will be "masked" because of the proportionately greater absorption of the widely spaced ions (see Results).

Because the distribution of dye in the system and therefore its metachromasy depends first of all upon interaction with the substrate, the factors which regulate dye-binding are of prime importance for metachromasy. For this reason the metachromasy of bound basic dye is in a sense a special case of basophilia. Among the important factors which control the binding of dye to substrate are pH, dye concentration, ionic strength, and the temperature of the dye bath. Each of these has been discussed in detail by one of us (Singer, 1952) and need not be repeated *in extenso*. Too often these factors have not been adequately considered. We shall return to this point when we consider the specific effect which has been attributed to sulfate, phosphate, and carboxyl groups.

In the combination of dye with substrate one ordinarily thinks of salt linkages (ionic or electrostatic bonds) providing the bond between the two. The controlling influence of pH and other factors which affect electrostatic forces in the staining reaction show that this is largely so. Yet, there is evidence to show that other bonds may operate alternatively or simultaneously in certain cases (Singer, 1952). Under certain conditions covalent links, hydrogen bonds, and van der Waal's forces may be involved in various ways. It is possible in the binding of basic dye under the conditions described above that, once coulombic forces are satisfied or relatively satisfied, other modes of linkage operate. Perhaps the development of metachromasy under these circumstances is due to such supplementary modes of attachment which intercalate new dye ions between satisfied ionic sites that are spaced more widely than 5 A. Another possibility of interest is that dye may be joined by some sort of association to bound dye to form aggregates which resemble those formed in solutions of the dye.

Some non-chromotropic tissue elements, like the red blood corpuscle, bind little basic dye and do not become metachromatic. Here the available groups appear to be too sparsely distributed. Certain other non-chromotropic tissue elements behave like fibrin. Thyroid colloid, smooth and skeletal muscle, as examples, develop metachromasy only when intensely stained. This degree of staining may be induced at high pH where most of the carboxyl groups become available. Like fibrin these tissue structures stain more intensely and become metachromatic more readily after formalin fixation; perhaps because the amino groups are covered by combination with formaldehyde and there is a relative increase in availability of negative binding sites. Nucleoproteins also become metachromatic with elevated dye-binding and the metachromasy is more easily obtained because of the ready availability and affinity of the phosphoric acid groups for the dye. The majority of these sites become available at a low pH in contrast to those of the carboxyl groups. Formalin fixation causes a less profound alteration in the basophilia and, therefore, in the metachromasy of nucleoprotein than in that of simple proteins because of the decided acid character of nucleoprotein, although this is less true for nuclear than cytoplasmic nucleoprotein (see Flax and Himes, 1952).

*The Color Destruction which is Inherent in Metachromasy:*

In addition to change in color, metachromasy also involves a notable decrease in color intensity, a fact which has been remarked upon repeatedly

for aqueous dye solutions (Fig. 2). It should also be stressed that in "successfully stained" tissue sections the extremely basophilic chromotropic elements, for example mast cell granules and cartilage matrix, are colored lightly, albeit metachromatically, in contrast to the obviously more intense orthochromatic color of less basophilic structures.

Michaelis (1947) suggested that the decrease in color intensity as metachromasy develops in aqueous solutions is due to the fact that clusters of dye ions permit more light to traverse the sample. This explanation makes the color reduction secondary—more apparent than real. The existence of an important primary effect upon color intensity is shown in the effect of dehydration upon the metachromatic films (Figs. 4 to 7). The dye distribution in the films is essentially invariant because of coulombic and other binding forces, yet desiccation produces a prominent increase in absorption as well as a return to the orthochromatic spectrum. Rehydration reverses these effects.

Two important conclusions can be drawn from these results; namely, that color destruction *in this absorption range* is a major intrinsic aspect of metachromasy and that water is the agent through which this occurs. This latter fact explains the otherwise enigmatic water dependence of metachromasy. It is the intercalation of water between suitably approximated dye cations, not the approximation *per se*, which causes metachromasy. This point will be considered in detail below in reference to the suggested mode of action of water.

*The Metachromasy of Stained Substrates, Relation to Metachromasy of Dye Solutions:*

The metachromasy of stained substrates is similar to that of concentrated aqueous solutions in color and in response to various environmental influences (temperature, solvent, salts, etc.). Consequently, a common explanation has been sought—to which some objections have been raised.

Michaelis (1944, etc.) attributed the metachromasy of chromotropes to the "adsorption of polymolecular dye micelles" similar to the polymers which exist in concentrated aqueous solution; but subsequently (1950) he questioned this view because agar stains metachromatically even under conditions of light staining when a monomolecular distribution over the anionic sites seemed to be the only reasonable assumption. Bank and Bungenberg de Jong (1939) and Wiame (1947) already had suggested that adjacently bound dye cations, instead of being polymerized directly to one another as in solution, may be aggregated or polymerized by way of the substrate. Recently Levine and Schubert (1952) were concerned with discrepancies in the behavior of the different absorption maxima as metachromasy develops in concentrated dye solutions with and without the chromotrope. Sylvén (1954) has pointed out the doubtful validity of such comparisons because of the heterogeneity of the sol systems. Recently, however, Schubert and Levine (1955) have proposed a polymer concept similar to the earlier Michaelis view.

The major spectral difference is that in chromotrope metachromasy the maximum ($\mu$ band) is displaced to lower wave lengths than the maximum ($\gamma$ band) observed in concentrated dye solutions. That this is a difference in degree rather than in kind of metachromasy is shown by the effect of dehydration on metachromatically stained films of the chromotropes (Figs. 4 to 7). The so called $\mu$ band gradually shifts bathochromically through the $\gamma$ band range to the position of the $\beta$ peak present in alcoholic and dilute aqueous solutions. Simultaneously, there is a great increase in total absorption which is proportionately greater in the $\alpha$ band position (see above). The same sort of response is obtained when the metachromasy of concentrated aqueous solutions is reversed by temperature, namely, a shift to higher wave lengths and an increase in total absorption favoring the $\alpha$ peak. As in the case of the heat effect, the effect of dehydration is reversible. The differences between chromotrope and solution metachromasy are thus quantitative rather than qualitative; the total change in absorption and the path traversed in shift between the position of the $\mu$ and $\beta$ bands are greater in chromotrope metachromasy. In the case of the metachromasy of non-chromotropes an intermediate quantitative situation prevails.

In concentrated aqueous solutions the observed color changes represent the distribution about some mean of a population composed of free ions and unstable aggregates of different sizes. In the stained chromotrope the distribution of the bound dye and its interaction are a function of the availability and distribution of binding sites in a three-dimensional solid system under the circumstances of the particular staining condition. The im-

portant consequence of the control over the distribution of the dye by the substrate is, as we have seen, that ion for ion the color intensity is proportionately lower and the color shift greater—a disparity which increases with the average number of associated ions. The special property of chromotropes is that practically all the available sites are suitably spaced and are frequently of the same kind; or that there is some other special structural relation that facilitates interaction of bound dye. The staining conditions, such as pH, exercise less control because of the favorable conditions for adjacent bonding and because most of the anionic sites become available at the same pH level. The evidence available shows that chromotrope metachromasy reaches a maximum when all sites are satisfied, the total absorption is minimal, and is shifted to the lowest wave length. In non-chromotropes, where the occurrence of properly spaced binding sites is not great and the pattern is not well defined, a suppression or delayed expression of metachromasy occurs. Moreover, the staining conditions exert a greater control in the development of metachromasy because they provide a means of binding tremendous amounts of dye and thus of increasing the chances that adjacent ions will be located within the 5 A minimum suggested by Sylvén for interaction.

There remains the question of specific effects of binding sites upon metachromasy. It is possible that the bond itself and the binding site may have some effect on color. Indeed the slight initial bathochromic shift observed when methylene blue is bound to fibrin seems to be due to some such effect. It is more difficult, especially in view of the role of water (see below), to understand a specific effect of the site on metachromasy. We have already pointed out that several investigators have reported that the sequence of sulfate, phosphate, and carboxyl represents the order of decreasing metachromasy. Sylvén (1954) asserts that this is true even if the spacing of sites is the same. For adequate comparison, however, proof of *equal saturation* as well as *spacing* of sites is needed. Actually, the sequence is in the order of dissociation of these three groups and therefore their availability at a given pH of staining.

In our opinion the metachromasy of dye solutions, of non-chromotropes, and of chromotropes is essentially the same phenomenon; the differences observed are mainly in degree. The evidence available indicates that ion for ion the total absorption decreases and the hypsochromic shift increases as the association number increases. In a specific situation the total absorption represents a summation of the absorption of the ion population. In solution, the dye population is not homogeneous; it is distributed about a mean association number. The position of the mean depends upon factors such as dye concentration, temperature, etc.

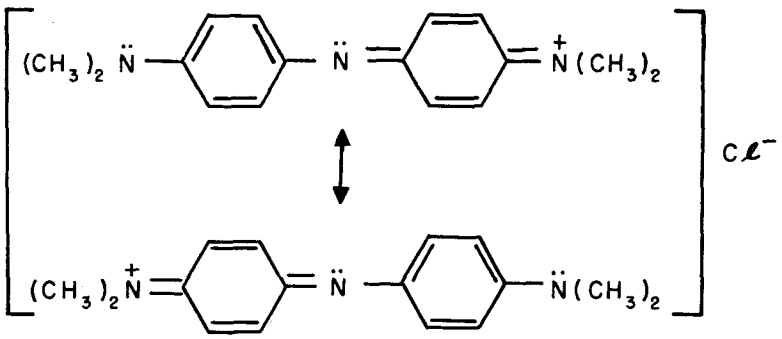
However, when dye interacts with a substrate, the internal pattern of satisfied binding sites determines both the form of the distribution and the mean or means. In the case of a chromotrope, for example the sulfate ester of a high molecular weight polysaccharide, the majority of the anionic sites are suitably spaced for interaction of adjacently bound dye and are similar. When saturation of these sites is approached, then most of the dye ions will be in the same state; there will be a homogeneous population of maximally metachromatic dye ions. A more complex situation exists in non-chromotropic substrates; the anionic sites can be of several sorts, the distribution is irregular and may have several maxima. Consequently, the factors which regulate the availability of binding sites play a more significant role in the development of metachromasy and the absorption changes which occur as saturation is approached are more complex.

Thus metachromasy in a particular substrate, chromotropic or not, is an expression of the pattern of binding sites.

*A Concept of Metachromasy:*

The goal of this speculation is a concept of metachromasy that is sufficiently comprehensive to illuminate the discussions above and to supplement the operational definition presented earlier (page 439). The foundations for this concept are two specific points established in this study, *namely that an essential feature of metachromasy is diminution in color intensity and that water molecules effect the interaction of suitably approximated dye ions.*

The point of departure is the view that the $\alpha$ and $\beta$ bands in the spectra of metachromatic dyes are the consequence of the resolution into two components of a single electronic band (see K. Venkataraman, volume 1, pp. 323–400, for general discussion on color and resonance). We shall illustrate this idea by reference to methylene blue. Bindschedler's green, a linear indamine dye, is non-metachromatic. The introduction of a sulfur linkage closing the internal ring converts it into the coplanar thiazine dye methylene blue which,



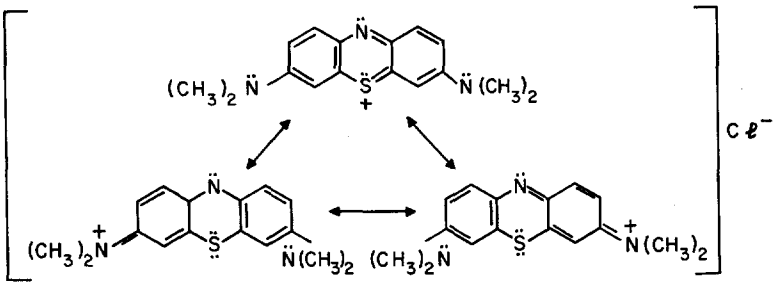

BINDSCHEDLER'S GREEN

FIG. 8. This non-metachromatic dye is the precursor of methylene blue. The linear cation is a resonance hybrid. The charge is free to move between the two terminal auxochromic groups.

spectrophotometrically speaking, is a striking metachromatic dye. We can attempt to analyze why this simple transformation endows methylene blue with properties that are lacking in Bindschedler's green. The green color of the parent dye can be attributed to the light absorption of an activated resonance hybrid in which the center of positive charge tends to be equidistant between the terminal auxochromes, the dimethyl amino groups (Fig. 8). On completion of the internal ring with the sulfur linkage there is a hypsochromic spectrophotometric shift; the new blue dye now absorbs at shorter wave lengths and the original simple spectrum of only one major peak in the visible range has been converted into two peaks, a major $\alpha$ peak and a minor $\beta$ peak (Figs. 1 to 3). The introduced sulfur can be considered to "compete" with the terminal auxochromes in establishing the redistribution of charge in the new resonance hybrid (Fig. 9). Following the terminology of Lewis and Calvin (1939) and Lewis and others (1942; 1943 *a* to *c*), the sulfur linkage introduces a center of asymmetry as the linear parent dye undergoes ring closure. As a consequence, there occurs the partial resolution of the single electronic band into two components. Specific evidence has been presented for this interpretation (Lewis *et al.*, 1939, 1942, 1943 *a* to *c*); for example, the fluorescence spectrum of methylene blue is the mirror image of the absorption spectrum (1943 *a*).

The intense, longer wave length of the $\alpha$ peak can be attributed (Lewis and others (1939, 1942, 1943 *a* to *c*)) to the interaction of the two powerful terminal auxochromes, while the less intense shorter wave length of the $\beta$ peak may be due to the interaction of the sulfur with these terminal groups. Stated otherwise, the $\alpha$ peak may reflect electron oscillations along the long or $Y$ axis, and the $\beta$ peak oscillations along the shorter or $X$ axis.

More support for this interpretation of the structural origin of the metachromatic potential may be drawn from the comparative spectroscopy of the thiazine dyes. Thionine, azure C, azure A, azure B, and methylene blue differ only in the degree of methylation of their terminal amino groups. Azure C is a monomethylated thionine, azure A is the asymmetrical dimethyl thionine, azure B is trimethyl thionine, and methylene blue is tetramethyl thionine. The absorption characteristics tend to vary in the following way; as methylation of the terminal amino groups increases, the distinction between the $\alpha$ and the $\beta$ peak becomes better defined and the color becomes more intense and bluer. That is, as the electron-donating potential of the terminal auxochromes becomes greater the discrepancy increases between their influence and that of the sulfur on the distribution of charge in the resonance hybrid. Consequently, the resolution into two components becomes clearer and the $\alpha$ peak in particular is strengthened. Ring closure in itself is not responsible for the metachromatic property. The same thiazine nucleus is present, for example, in the imine bases of thionine and the azures. These imine bases are not metachromatic although their color approaches the metachromatic spectrum. It is the asymmetry introduced on ring closure which is responsible for the metachromatic potential (see below). Inspection shows that all



METHYLENE BLUE

FIG. 9. The introduction of sulfur converts the linear cation of Bindschedler's green into the planar methylene blue cation. The new dye is a resonance hybrid of three main structures. The charge can move in a plane over paths of different character.

the recognized metachromatic dyes possess this partial resolution of a single electronic band into two components.

We indicated earlier that Sheppard and Geddes (1944) took issue with the popular view that the so called dimer band ($\beta$) is a new one. They pointed out that this band arises at the site of a "bump" which is present both in extremely dilute aqueous solutions and in alcoholic solutions. As we have done here, they attributed this "bump" in quasi-classical terms (Lewis and Calvin, 1939) to the partial resolution of a single electronic band. This "bump" gives rise to a prominent band which is not new but represents ". . . vibrationally coupled transitions proper to the monomer ion, which, however, are enhanced in the dimer." Michaelis (1947) subsequently accepted this interpretation of the $\beta$ band.

We have seen that metachromasy involves a diminution of color and that the elevation of the $\beta$ band is only apparent. It is due to the proportionately greater reduction of the $\alpha$ band. Some interference seems to occur which depresses the resonance of the dye ions but affects the $\alpha$-band ($y$ component) more than the $\beta$ band ($x$ component). In more refined terminology, the constraint is directed at the $\pi$ electrons in level (N/2). A more complex situation exists in high polymers which also have a $z$ component. In the interpretation of this effect it is important to consider the role of water. Sheppard and Geddes (1944) proposed that water acts as an electronic buffer, permits the approach of the cations, and is thus essential for aggregation. While not disputing this possibility, the results presented above require a more direct role of water in color determination because, whatever the forces of approximation, approximation *per se* does not produce metachromacy, but the presence as well of water does.

An intimate association of the dye with water was suggested by Sheppard and Geddes (1944) for the following reason. The sublimate of one dye remained metachromatic when dissolved in absolute alcohol. The addition of water up to 3 per cent abolished the metachromasy. Apparently, the water had "frozen in" and could be displaced by more water but not alcohol. We have already noted the difficulty in dehydrating metachromatically stained films and tissues. This is also true of crystalline metachromatic dyes. The solid powders contain water and are metachromatic, a fact which is generally not appreciated. A temperature of 150°C. is needed to dry methylene blue completely and to abolish therewith the metachromatic color.

One mechanism comes to mind by which water molecules might interfere with the resonance of approximated dye ions and affect the $\alpha$ band more than the $\beta$ band, *i.e.* produce metachromasy. We will note at this juncture that this mechanism can influence only basic dyes, as a general rule, because only in these is the charge mobile and directly involved in color. The negative charge of acid dyes is restricted to groups which generally do not contribute greatly to the color of the dye. As coplanar dye cations approach each other and enter the range where charge repulsion becomes increasingly strong, the mobile charge will tend to redistribute in a manner that minimizes the repulsion. This would be towards opposite ends. If two such dye ions with redistributed charge "stacked" very closely, coordinate or dative bonds could form between the terminal auxochromes by the shifting of two loosely bonded electrons from

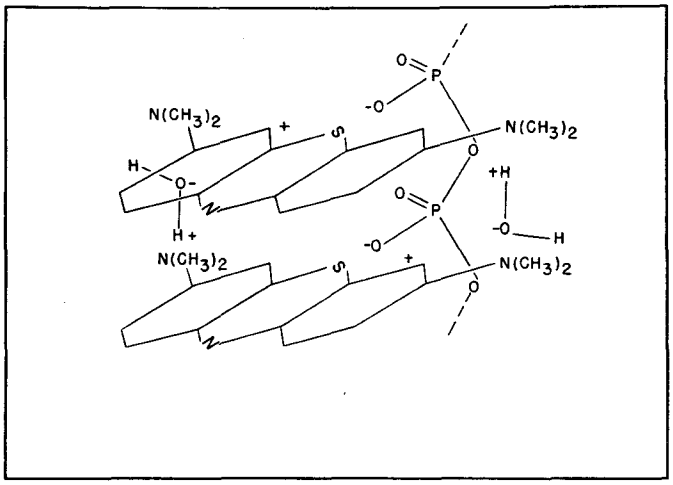



FIG. 10. A schematic representation, in three dimensions, of two methylene blue cations which are oriented at adjacent anionic sites of a phosphate polymer. The dipolar nature of the intercalated water molecules is emphasized to suggest interaction with the terminal auxochromic groups.

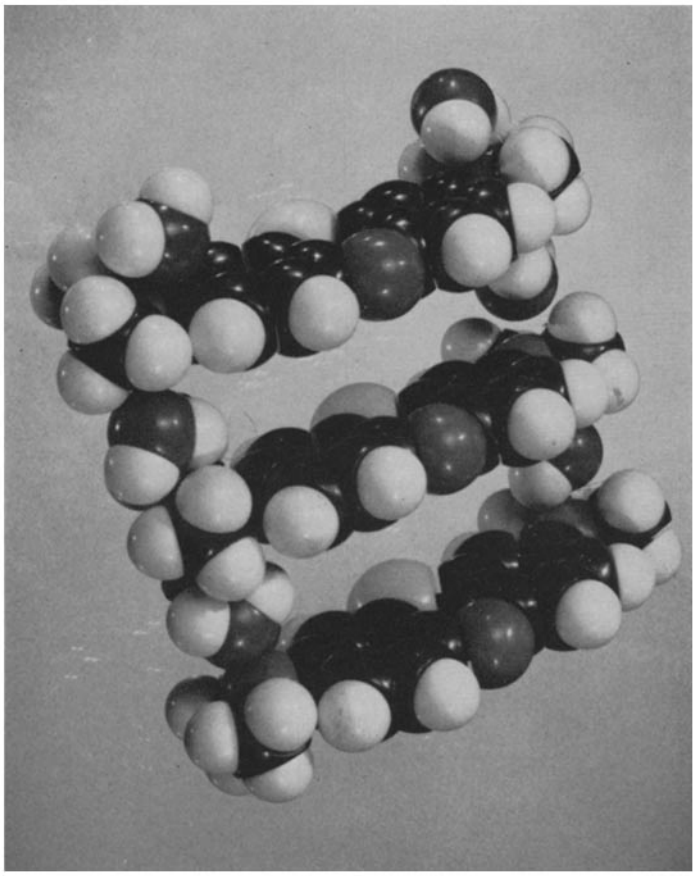


FIG. 11. Molecular models of the Stuart type. An aggregate of three methylene blue ions separated by 7 A to show the oriented, intercalated water molecules.

the negative nitrogen of one to the approximated positively charged nitrogen of the other, the reverse occurring at the opposite end. A doubly charged complex would result in which electron mobility, especially the electron-donating character of the terminal auxochromes, would be greatly depressed. We *do not* entertain this possibility as the explanation of metachromasy but rather that water, a strong dipole, will orient between such groups and will have a depressing effect on the resonance.

This is illustrated in Figs. 10 and 11. Fig. 10 is a schematic representation in which two ions of methylene blue are electrostatically attracted to a polymeric metaphosphate. Fig. 11 employs molecular models at a separation equivalent to 7 A. At closer spacings the water molecules tend to be obscured. The water influence would tend to be reinforced as the number of associated ions increases. Any factor (temperature elevation, solvents, etc.) which displaces water or separates the ions will effectively reduce the metachromasy. Tentatively, this simple model seems to offer a comprehensive explanation of metachromasy.

The model proposed also sheds some light on the reason for the long standing controversy whether methylene blue is really a metachromatic dye (see introduction). Methylene blue was rejected for two major reasons, the practical fact that the color change is not obvious or striking to the eye and the observation that its properties could not be fitted along with those of other metachromatic dyes into a uniform hypothesis of metachromasy. Instead, other explanations were offered for its color change such as chemical change into a derivative of different color. Michaelis (1902 *a*) was the first to eliminate methylene blue from consideration for such theoretical reasons. Years later (1947), however, he employed the dye almost exclusively in his studies of metachromasy because spectrophotometrically its metachromasy is the best defined of the thiazines.

The answer to the paradox is that the $\alpha$ peak of methylene blue depresses more slowly and less completely than the $\alpha$ peak of the less methylated homologues. As a consequence, the color shift is not as noticeable to the naked eye, although the separate behavior of the peaks is sharply defined spectrophotometrically. This behavior is reasonable in terms of the model proposed because the interference is proportionately smaller the stronger the terminal auxochromes.

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