[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH, NEW YORK]

Metachromasy of Basic Dyestuffs

BY L. MICHAELIS AND S. GRANICK

The absorption spectrum of a dyestuff is not necessarily correlated with its chemical structure alone. Sometimes even the solid, crystalline dye may exist in two modifications of different color. In the dissolved state, the absorption spectrum usually depends, to some extent, on the nature of the solvent. Often it depends on concentration.[1] In this paper another variability of color will be discussed, namely, the variability of color of an adsorbed dye, as depending on the nature of the stainable substrate at which it is adsorbed. This effect, termed metachromasy by P. Ehrlich, has been known for a long time to histologists and has been utilized in histological staining methods. In this paper the discussion will be restricted to basic dyes. Although similar phenomena may occur also for acid dyes, the underlying principles are not always the same. As regards basic dyes, a certain correlation of their metachromatic faculty with their variability of color in aqueous solution will be demonstrated.

If the molar absorption coefficients plotted against wave lengths, for any molecular species dissolved in a given solvent, yield a curve which is independent of the concentration of the solute, such a substance is said to obey Beer's law. Various papers[2,3,4,5] agree on the fact that the majority of organic dyestuffs do not obey Beer's law, the aberrations varying within wide limits among various dyes. It is generally agreed that such deviations are due to a reversible polymerization of the dye molecules, the polymers exhibiting an absorption spectrum different from that of the monomers. It is also agreed that in most cases the polymerization of basic dyes is, at least essentially, restricted to dimerization, although in some cases, to wit for polymethine dyes, according to Scheibe,[3] high polymers are also readily formed in aqueous solution of sufficiently high concentration. Although the nature of the attractive force ensuing in molecular aggregation seems understandable in a general, qualitative manner, no satisfactory quantitative theory has been established yet. Attempts in this respect, as made by Rabinowitch and Epstein,[5] do not account for the large differences with respect to the aberrations from Beer's law which are sometimes encountered for dyestuffs of closely related chemical structure, and of very similar absorption spectra in their monomeric state. To give a few striking examples: on the one hand, thionine exhibits large deviations from Beer's law in aqueous solution and, on the other hand, oxonine, which differs from it only by the substitution of an S bridge by an O bridge, and exhibits, in dilute solution, almost the same absorption spectrum (peak for thionine at 600 mμ, for oxonine at 580 mμ), shows only extremely small aberration from Beer's law. The deviations are large for methylene blue, very much smaller for capri blue, and not noticeable at all for Bindschedler's green. It is not the aim of this paper to enter upon a discussion of the nature of the associating force, rather will it be shown that the varying ability of dyestuffs for dimerization in aqueous solution goes hand in hand with their varying metachromatic effect exhibited in certain staining processes.[6,7,8] The matter will be presented essentially in a descriptive manner, leaving the establishment of a rational theory to further study.

The best known metachromatic basic dyes are toluidine blue and thionine. They stain some histological morphological elements, such as cellular nuclei or the cytoplasma of lymphocytes, blue, which may be called the "normal" color because it resembles that exhibited by a dilute aqueous solution of the dye.[9] Some other morphological elements are stained purple, more pink or more violet according to conditions. This is the metachromatic color. It should be stated right here that the normal color obtained by staining nucleic acid with any one basic dye is one definite, invariable color, while the shade of the metachromatic color is somewhat variable according to conditions. Lison[7] has shown that all distinctly metachromatically staining substrates, at least all those which may occur to histologists, are, or contain, half-esters of sulfuric acid with a high-polymeric carbohydrate. Among those are: cartilage and chondroitin–sulfuric acid; "amyloid"

(1) Variability of color due to change of *p*H is not included in the discussion. Here change of color is indicative of an obvious change in chemical composition of the molecule, such as the loss of a proton.

(2) V. L. Lewshin, *Acta Physicochem.*, U. S. S. R., **1**, 685 (1935); **4**, 221 (1936).

(3) G. Scheibe, *Kolloid Z.*, 82 (1938).

(4) W. C. Holmes, *Ind. Eng. Chem.*, **16**, 35 (1924).

(5) E. Rabinowitch and L. F. Epstein, THIS JOURNAL, **63**, 69 (1941); G. N. Lewis, M. T. Magel and D. Lipkin, *ibid.*, **64**, 1778 (1942); S. E. Sheppard and A. L. Geddes, *ibid.*, **66**, 1995 and 2003 (1944).

(6) P. Ehrlich, *Arch. mikroskop. Anat. Entwicklungsmech.*, 13 (1877); L. Michaelis, "Einführung in die Farbstoffchemie," S. Karger, Berlin, 1902, p. 116; W. and M. Moellendorff, *Ergeb. Anat. Entwicklungsgeschichte*, **25**, 1 (1924).

(7) L. Lison, *Arch. biol.*, **46**, 599 (1935); L. Lison, *Protoplasma*, **24**, 453 (1935); L. Lison, "Histochimie animale," Gauthier, Paris, 1936.

(8) A. T. Gzaja, *Planta*, 11 (1930); 21 (1934); 24 (1935), and 26 (1936); O. Bank and H. G. Bungenberg-De Jong, *Protoplasma*, **33**, 489 (1939).

(9) Moellendorf (*cf.* ref. 6) emphasized that also cellular nuclei stained with highly concentrated dye solutions, may be slightly metachromatically stained. It is likely that this phenomenon is correlated with that peculiar staining effect of chromatin which is known as the "Romanowski effect" and usually obtained by what is called the "Giemsa stain." Although this may be true for nucleoprotein as existing in cellular nuclei, it is not true for nucleic acid itself. In what follows the staining properties of the whole nucleus will not be considered but only those of nucleic acid.

substance occurring pathologically in many organs; muco-proteids and mucoitin-sulfuric acid; the basophilic granulae of certain leucocytes (Ehrlich's "mast cells"), now identified with heparin, which according to Jorpes[10] is also such a sulfuric ester; and a number of vegetable colloids listed in Lison's monograph, among which agar is the most readily accessible. It is, according to Neuberg,[11] the calcium salt of a sulfuric ester of a high-polymeric carbohydrate.

In order to study the color spectrophotometrically, the stainable substrate is best chosen in the form of a colloidal macro-homogeneous, clear, or even turbid solution. Nucleic acid has been chosen as a model for a normally staining substrate, and agar for a metachromatically staining one. As regards nucleic acid, no difference was observed whether a clear solution, at *p*H 4.5 to 5, of a low molecular weight yeast nucleic acid, or a turbid solution of a specimen of a high-polymeric thymonucleic acid was used. Agar was used as an aqueous gel, also usually at *p*H 4.5 to 5. When such a colloidal solution of the stainable substrate is mixed with a basic dye, the result depends on the concentration of both components. When a relatively highly concentrated dye solution is mixed with very little of the substrate, an amorphous precipitate arises, of normal color with nucleic acid, and of metachromatic color with agar. When a relatively highly concentrated solution of the substrate is mixed with little dye, no visible precipitate is formed due to the highly viscous nature of the solvent. This latter condition is best suited for spectrophotometric measurements. It will be shown with this method that the degree to which a dye exhibits metachromatic properties in agar goes parallel to the degree to which it deviates from Beer's law in aqueous solution. The color obtained with nucleic acid, however, is independent of this property.

For dyestuffs which in alkaline solution can form a free uncharged base (namely all those dyes which are not quaternary bases), the metachromatic color resembles somewhat that of the free base. However, since the metachromatic effect is nearly independent of *p*H, at least within the *p*H range from 3 to 8, and is established at a *p*H incompatible with the existence of the free bases for almost all the dyes investigated, metachromasy cannot be attributed to the formation of the free base. Nor is the metachromatic color spectrophotometrically the same as that of the free base.

All metachromatic effects are diminished or even abolished, reversibly, on increasing the temperature. On decreasing the temperature, the establishment of the final state of color may sometimes lag behind the drop in temperature, but the final state indicates a perfect reversibility. In many cases the metachromatic color can be readily distinguished from the normal one by the unaided eye, in other cases the contrast is less conspicuous to the eye but just as striking spectrophotometrically, for instance with methylene blue. Again, in other cases, metachromasy is not exhibited even spectrophotometrically, namely, for dyestuffs which obey Beer's law in aqueous solution, such as methyl green or malachite green.

When to a very dilute dye solution of metachromatic dyes, ammonium sulfate is added, a shift of the absorption spectrum takes place, which resembles the metachromatic effect. The degree of this effect depends on the concentration of the salt. It never reaches the same extent as is obtained with agar. Obviously a salting out effect takes place which results in a relatively stable colloidal solution, and the shift of the absorption spectrum is due to the formation of molecular aggregates.

Spectrophotometrically, the metachromatic effect exhibited by the dye adsorbed on agar manifests itself by a strong displacement of the absorption band toward shorter wave lengths. The main, or α, band of the dye is strongly depressed or even suppressed at the expense of the new "metachromatic" band. In principle, it is the same effect as that of a high concentration of the dye in aqueous solution, where the α band is depressed at the expense of the dimeric, or β, band. The difference is that the metachromatic band is much farther displaced toward the violet than is the dimeric band. Furthermore, whereas for all dyestuffs investigated here the peak of the β band for any one dye seems to lie always at the same wave length, independent of concentration,[12] at least in such a concentration range as is accessible to quantitative measurement, and seems always to coincide with that little hump of the α band which appears even in infinitely dilute aqueous solution and even in alcohol solution, the metachromatic band does not always lie at the same wave length but depends somewhat on the dye concentration. The displacement toward violet is stronger with increase in dye concentration and, with increasing concentration, reaches a limit asymptotically. In all probability the metachromatic band may be interpreted as due to high polymers. For polymethine dyes Scheibe[3] has

(10) J. Erik Jorpes, "Heparin," Oxford University Press, 1939.

(11) C. Neuberg and Ohle, *Biochem. Z.*, **125**, 311 (1921).

(12) This statement is in some respects in contrast to Lison's (*cf.* ref. 7) observations. This author was the first to notice the parallelity, for a basic dye, between the disobeying of Beer's law and metachromasy. However, he claims that a gradual increase in concentration in aqueous solution continuously shifts the peak of absorption toward the violet. This statement has not been confirmed by any other author for any one of the dyes, at least those of the type investigated here. In aqueous solution the maximum of the α band, and that of the β band for any one dye always lie each at one definite wave length (except perhaps for extremely high concentrations—Fig. 1), at least within the limits of error caused by the overlapping of the two bands. Essentially only the relative heights of the two bands are changed by varying the concentration. In agar, however, the metachromatic band does not lie at one definite wave length, but is the more displaced toward the violet the higher the dye concentration.

observed a progressive displacement of the band with increasing concentration even in aqueous solution. In the presence of ammonium sulfate such a progressive displacement takes place but to a lesser extent than in agar. The metachromatic band is lower at its maximum, and more diffuse than the monomeric band. Not only is the peak of absorption lower, but, in spite of its greater diffuseness, the absorption integrated over the whole visible spectrum seems to be smaller than that of the α-band as it appears in a dilute aqueous solution.

In contrast herewith is the behavior of the dye in nucleic acid. When a 3% solution of nucleic acid at *p*H about 4.6, is mixed with the dye, the color is similar to although not quite the same as that in dilute aqueous solution. The difference is that the peak of absorption is slightly displaced toward *longer* wave lengths by nucleic acid, the peak of absorption slightly lowered, and, what is most important, the molar absorption curve of the dye in the presence of excessive nucleic acid is independent of the dye concentration: Beer's law is strictly obeyed. Nothing reminding one of the dimeric band in aqueous solution can be observed on increasing the dye concentration. Those slight changes, both in the location and in the height of the band, brought about by nucleic acid are in opposite direction from those slight changes brought about by taking alcohol as solvent instead of water. The absence of aberrations from Beer's law in nucleic acid recalls the absence, or at least the considerable diminution, of the aberrations in alcohol. All basic dyestuffs investigated behave in the same manner toward nucleic acid, whether or not they are metachromatic with agar and whether or not they disobey Beer's law in aqueous solution.

When a 10% gelatin gel, at *p*H 4.6, is mixed with a basic dye, the absorption spectrum is precisely the same as in aqueous solution at the same dye concentration, including also the appearance of the β band at increased concentration. This substrate obviously does not adsorb the dye to any appreciable extent. It is shown hereby that the mere colloidal or gelatinous state of the medium is irrelevant for the absorption spectrum of the dye.

The Ultraviolet Band

All those dyestuffs have in addition to the absorption band in the visible, another in the quartz ultraviolet, the maximum of which is sometimes very much lower than that of the visible band (oxonine, Bindschedler's green) sometimes, although lower, yet of comparable height (thionine, methylene blue) and sometimes as high as the visible band (phenosafranine, trypaflavine). The problem now arises whether this ultraviolet band is variable in a way similar to that of the visible band. It has been known that the ultraviolet band is practically independent of the concentration of the dye even in such cases where the visible band disobeys Beer's law. This fact is corroborated by the present studies and amplified by the statement that even in the presence of agar the ultraviolet band is not affected. The great variability of the visible band is not exhibited by the ultraviolet band. For any theory concerning the nature of the ultraviolet band of dyestuffs, this fact must be borne in mind.

Other Types of Metachromatic Effects

Variability in color of a dyestuff may be encountered not only when the dye is adsorbed on stainable substrates of various kinds, but also on other occasions. A few examples of this kind will be mentioned, although no exhaustive description of the phenomena is intended here. A metachromatic dyestuff can exist in the solid state in two modifications, one with color strongly resembling the normal, another with metachromatic color. When a solution of toluidine blue, thionine or methylene blue is salted out, for instance, with sodium perchlorate, the precipitate is pinkish violet, at first amorphous but gradually, more or less readily according to conditions which are not easy to control, becoming crystalline, forming pink-purple needles. Gradually in time, or more rapidly on recrystallization from the hot solution, intensely blue needles are formed. The latter form, of normal color, is the stable one. It is the form generally known for the solid dyes. When the hydrochloride of oxonine is salted out by sodium chloride, blue-black crystals are formed. The perchlorate, as it crystallizes from hot 50% alcohol, forms brownish crystals (dichroitic dark brown and light yellow). Even Bindschedler's green, for which there is no evidence of metachromatic staining, crystallizes in two forms of the zinc chloride double salt, either green or red, according to Wieland.[13] Such examples show that the absorption spectrum of one molecular species may be greatly modified according to the spacial mutual orientation of adjacent molecules.

Discussion

The nature of the "normal" coloring effect of a basic dye with nucleic acid may be inferred from the fact that the dye, when combined with nucleic acid, strictly follows Beer's law, in contrast to its behavior in aqueous solution. The dye–substrate compound is obviously unambiguously defined independent of the dye concentration. The adsorbed dye molecules do not interact with each other optically in any way depending on their concentration. Obviously, a stoichiometrically unambiguously defined, salt-like compound is formed from each cation of the dye with one acidic side chain of nucleic acid. Metachromatic staining exhibited in agar cannot be of the same nature. Here obviously the dye is precipitated so as to form molecular aggregates, the molecules of the aggregates being spacially

(13) H. Wieland, *Ber.*, **48**, 1087 (1915).

arranged in such a way as to bring about optical interaction. It is those molecular aggregates which are adsorbed by the negatively charged side chains of agar. Here also the opposite charge of the dye cation and the agar micelle is involved in the adsorption, since only basic dyes are adsorbed by agar. No acid dye is able to stain substrates such as agar or basophilic granulae of leucocytes. However, it is not a single dye cation which is adsorbed by each one negatively charged side chain of the substrate, but an aggregate. It is hard to imagine that such aggregates of the dye molecules should not include also inorganic anions. One may imagine that one cation of the dye is adsorbed primarily by an acid side chain of the substrate and carries along an aggregate of other dyestuff molecules including their anions. The fact that metachromatic color in agar gives way to an approximately normal color at higher temperatures in a reversible way corroborates the hypothesis of polymerization of the dye molecules. The fact that the peak of absorption for the metachromatic color in agar is not at one constant wave length, but is more displaced toward violet with increasing dye concentration, shows that the dye–substrate compound is not an unambiguously defined compound as in nucleic acid, but variable.

Staining of nucleic acid is a prototype of an exchange adsorption, a cation of the substrate being exchanged by a dye cation as in the permutite reaction. A comparison of the staining of nucleic acid and of agar shows that not all staining effects are quite of the same nature, not even those in which the opposite electric charges of the dye and the substrate are primarily involved in the process of adsorption.

Experimental Part

All dyestuffs were pure crystalline preparations, in part prepared in this Laboratory, as described in previous papers, in part standardized commercial preparations. The latter are rarely ash-free and cannot be used directly for the determination of absolute values of molar absorption coefficients. Corrections were made by converting the dyestuffs into their sparingly soluble and readily crystallizable perchlorates, which, after being dried at 100°, and dissolved in alcohol to a concentration of the order of 10^{-6} M, were spectrophotometrically compared with alcohol solutions of the stock preparations, relying on the fact that in very dilute alcohol solutions Beer's law is valid. The perchlorates themselves are, as a rule, not sufficiently soluble in water to allow the study of widely varied concentrations. Beckman's photoelectric spectrophotometer[14] was used, with absorption vessels of thickness from 1.00 cm. down to 0.0566 cm.,[15] the latter being calibrated optically with solutions of potassium ferricyanide which strictly obeys the Beer–Lambert law (this is not true for potassium bichromate which is sometimes recommended.) In the definition of the molar absorption coefficient the thickness of the cell is measured in cm., the concentration in moles per liter, and common logarithms are used. Only a few examples of the experimental material are being presented here.

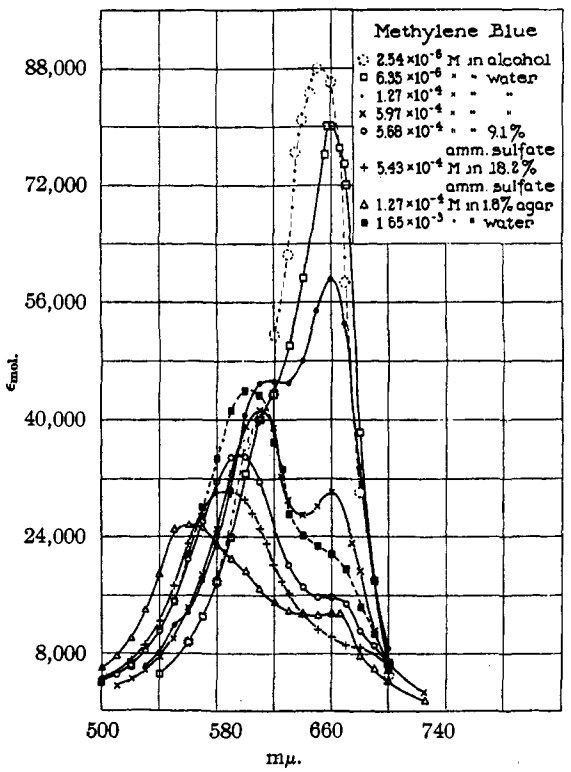

Fig. 1.

Thiazines and Oxazines: Methylene Blue.—In Figure 1 the molar absorption curve is shown, first in alcohol. The maximum lies at 650 mμ, and ϵ_{mol} amounts here to 88,000. These values are practically independent of the concentration (not shown in the diagram), at least within the range of low concentrations. In aqueous solution the peak of ϵ_{mol} is somewhat lower, even at highest dilution, and lies at 658 mμ. With increasing concentration this maximum is lowered without being displaced, and a second band, the β band, arises, with peak at 615 mμ,[16] which on further increase of concentration becomes stronger, at the expense of the first band, which eventually may be almost suppressed. Only for the very highest concentration, is the β band slightly displaced farther toward violet. The α band at 658 is characteristic of the monomer, the β band at 615 of the dimer. It should be noticed that the monomeric band shows a slight hump at the wave length of the dimeric band. In ammonium sulfate solution the second band is displaced farther in the direction of shorter wave lengths, and in agar this displacement reaches 560 mμ. At the conditions prevailing in this experiment with agar the α band is almost, although not entirely, suppressed. The displaced band is interpreted as due to higher polymers.

Toluidine Blue (Fig. 2).—In alcohol, ϵ_{mol} has its peak at 630 mμ and amounts to 63,000, practically independent of the dye concentration. In aqueous solution two curves are shown for different concentrations. Even at the lowest concentration shown here, a β band, overlapping with

(14) H. H. Cary and A. O. Beckman, *J. Optical Soc. Am.*, **31**, 682 (1941).

(15) The narrow cells were obtained by using a quartz cell 0.5 cm. thick with a parallelopipedic quartz inset to reduce the thickness. In a few experiments a still narrower cell than that indicated above, namely 0.00578 cm. (a value reproducible in independent calibrations within a few per cent.) was used.

(16) According to Sheppard[6] the "dimeric" band is not a new band, but due to the fact that certain bands, involving transitions to various vibrational states which are forbidden in the monomolecular state, are enhanced in the polymeric state. This problem will not be discussed here, and the term "dimeric" band will be permissible even if Sheppard's hypothesis be accepted.

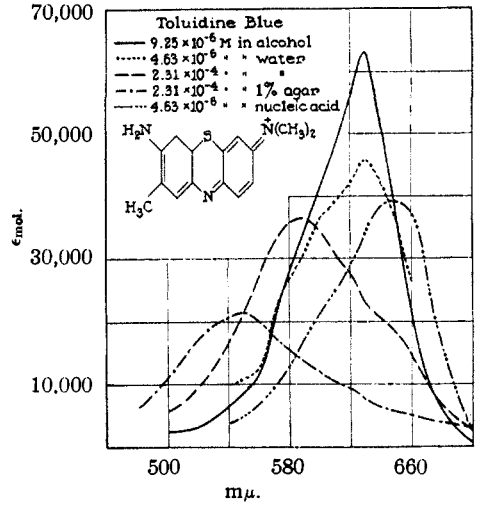

Fig. 2.

the α band, is established at 590 mμ. In agar the band is very diffuse, the maximum being displaced to 550 mμ. In nucleic acid there is only one band, comparable to the α band in aqueous solution, slightly displaced toward longer wave length.

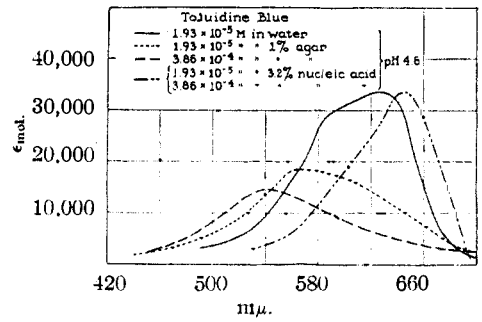

Fig. 3.

Figure 3 shows that the agar band is displaced depending on the dye concentration, either to 560, or to 540 mμ. The latter may be considered as the limit of attainable displacement. On the other hand, the band in nucleic acid is independent of the dye concentration, no β band is developed on increasing the concentration.

Figure 4 shows that ϵ_{mol} of **oxonine** in aqueous solution does not depend to any degree safely beyond the limits of experimental error, on the concentration within those limits where the two previous dyes show large deviations. Only at a very much higher concentration does a β band develop. In agar also, in this diagram, no change of the curve can be seen. However, after standing for more than an hour, a slight band forms at 460 mμ, as shown at an enlarged scale in Fig. 5. This "metachromatic band" can be more readily seen on using a much higher dyestuff concentration and observing a thin film of the agar spread on a slide, with the hand spectroscope. This metachromatic band disappears reversibly on heating. This dyestuff, which differs from the strongly metachromatic thionine only by a substitution of the S atom by an O atom, is metachromatic only to a just noticeable extent and deviates from Beer's law only to an extremely small extent.[17]

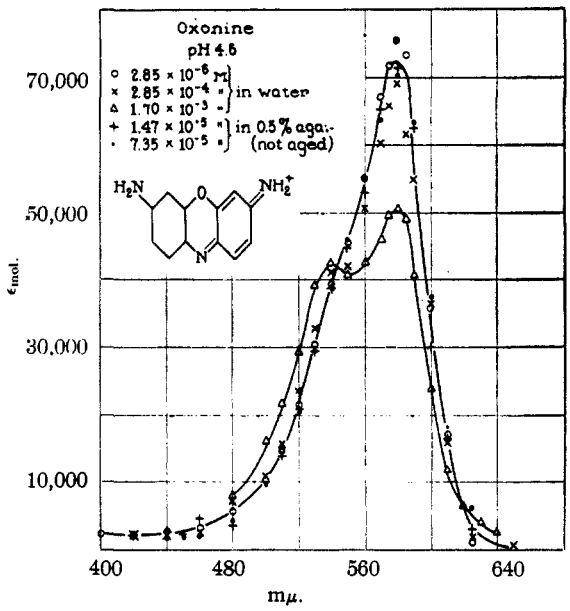

Fig. 4.

Thionine behaves very much like toluidine blue. It should be noticed that the substitution of the S bridge of thionine by O in oxonine almost destroys the faculty of dimerization or polymerization, even to a higher extent

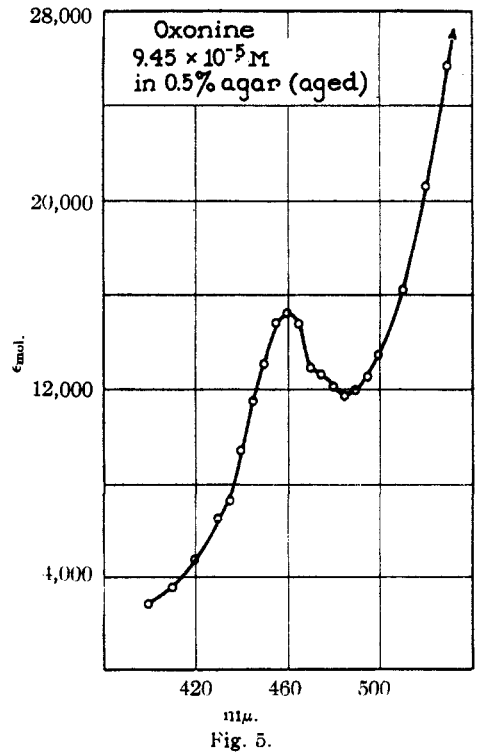

Fig. 5.

(17) One may demand that the degree of deviation from Beer's law should be expressed quantitatively. Rabinowitch and Epstein[5] did so by calculating the dimerization constant from spectrophotometrical data, using the mass action law. Neither Sheppard[6] nor the writers succeeded in obtaining such constants satisfactorily. This may be due to the fact that the extrapolation necessary for such calculations are not safe enough, or that the mass action law, in terms of uncorrected concentrations, is not valid, or perhaps even because it is not strictly true that no higher polymers are formed. In any case, this uncertainty prompted the writer to express the degree of deviations from Beer's law among various dyestuffs in less rigid form, which, however, is sufficient for the purpose.

than was described previously[18] with a less precise method. The contrast between thionine and oxonine in this respect clearly shows the deficiency of any present theory which attempts to correlate the tendency of polymerization with chemical structure. For **selenium methylene blue** the deviations from Beer's law are somewhat greater than for methylene blue, and so is the metachromatic effect.

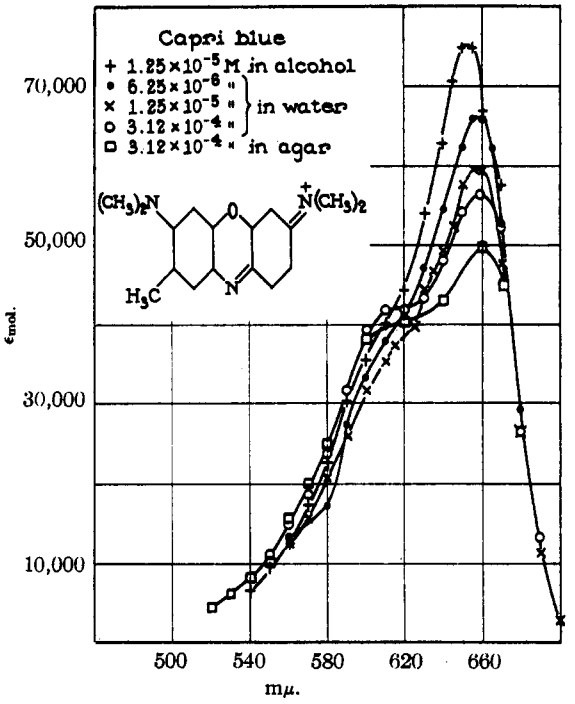

Fig. 6.

Figure 6 shows that in **capri blue** the effect of higher concentration in aqueous solution is, although not entirely missing, yet much smaller than for the thiazine dyes. Even in agar only a slight decrease, but no distinct displacement, of the β band can be seen. On the other hand, Fig. 7 shows that the absorption curve in nucleic acid has the same character as that obtained with the thiazine dyes. Hence, capri blue behaves like methylene blue with respect to its "normal" staining effect in nucleic acid, but differs greatly from it with respect to its metachromatic potency in agar.

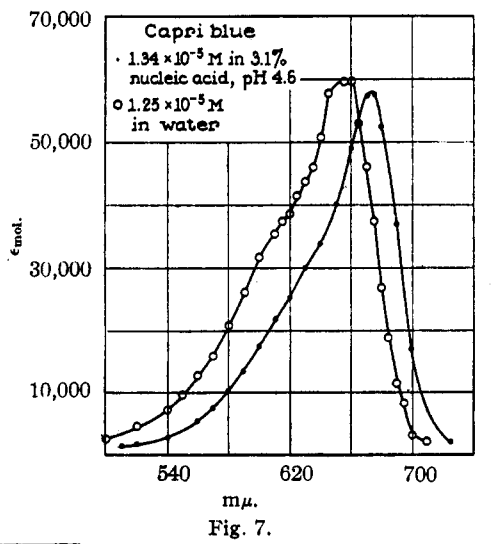

Fig. 7.

Nile blue, another oxazine dye, which however differs from the other in that it contains a naphthalene ring, approaches the characteristics of the thiazine dyes. There is

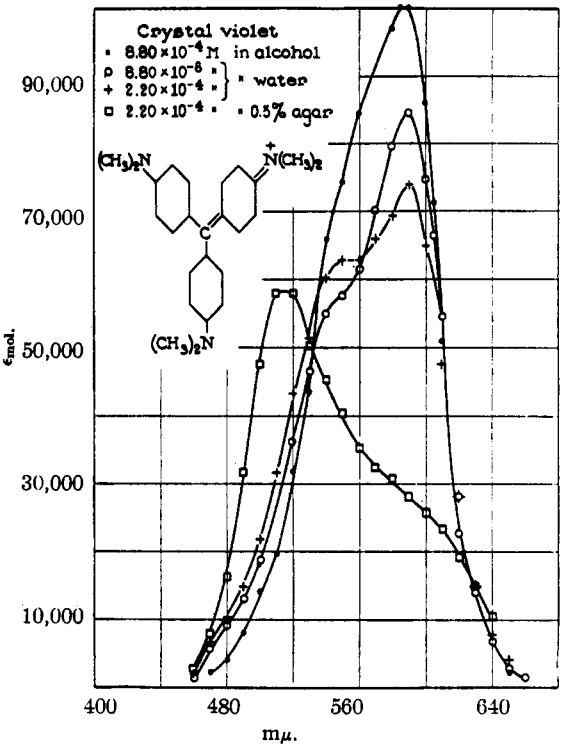

Fig. 8.

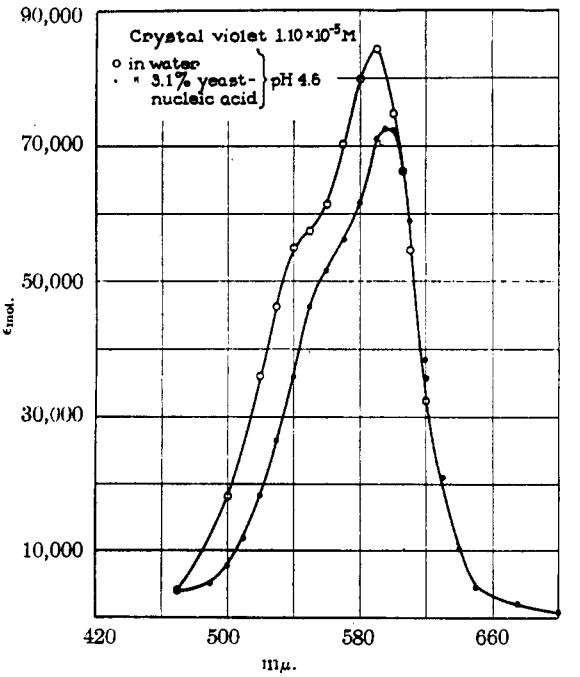

Fig. 9.

(18) S. Granick, L. Michaelis and M. P. Schubert, THIS JOURNAL, **62**, 1802 (1940).

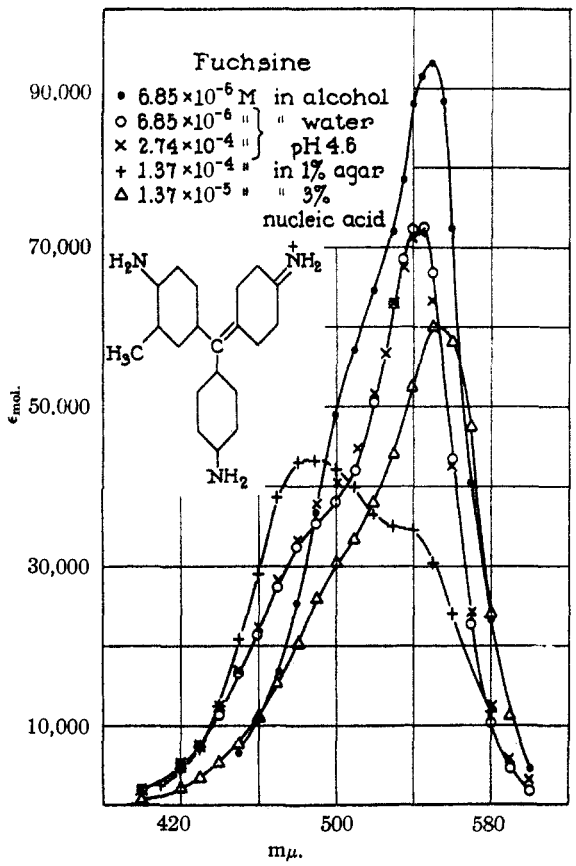

Fig. 10.

a distinct although relatively week dependence of the curve on concentration in aqueous solution, and a distinct displacement of the β band in agar.

Triphenylmethane Dyes.—For **crystal violet** Fig. 8 shows a distinct, although relatively small, dependence of the curve on the dye concentration in aqueous solution and a farther displacement of the maximum in agar, from 590 to 495 mμ. Figure 9 shows that the "normal" color developed in nucleic acid is of the usual type.

In **fuchsine** (Fig. 10) no distinct change of the curve with increased concentration, at least in the usual range, in aqueous solution can be seen. In agar there is a distinct displacement, although to a smaller extent (from 543 to 485 mμ). The "normal" color in nucleic acid is of the usual type.

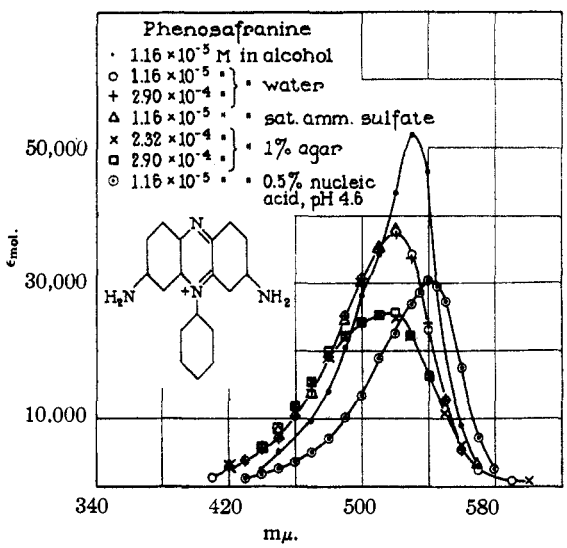

Fig. 12.

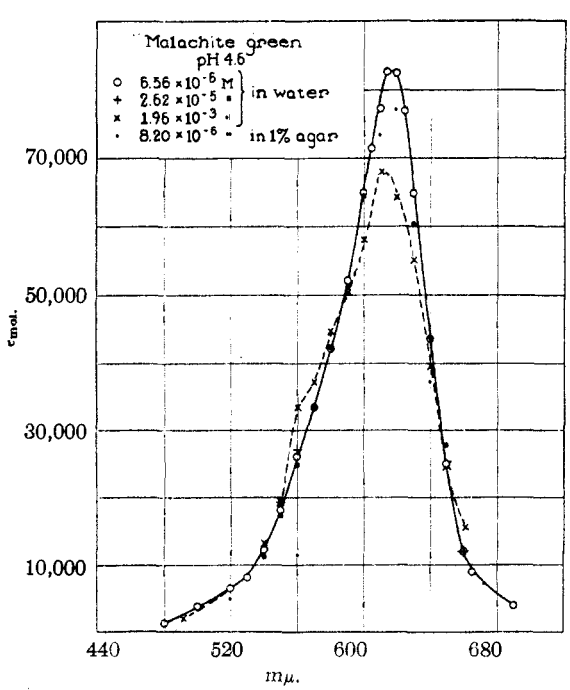

Fig. 11.

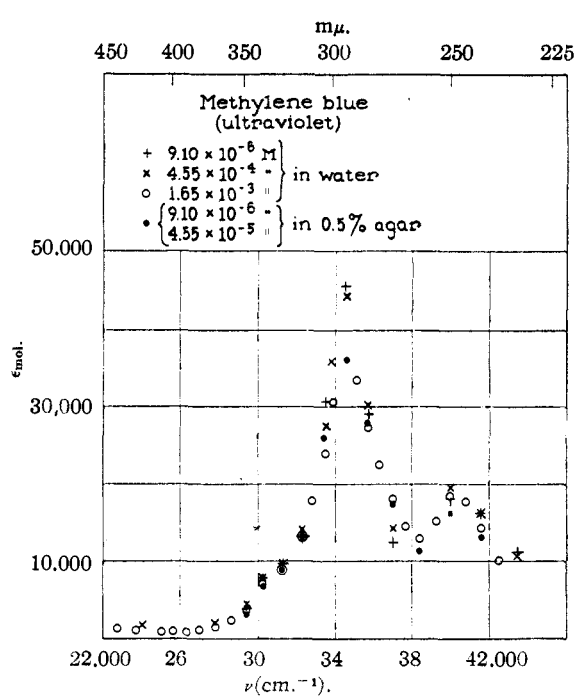

Fig. 13.

For **malachite green** (Fig. 11) no influence of varied concentration and only an extremely small, perhaps doubtful one, in the presence of agar, has been observed.

In **phenosafranin** (Fig. 12) no influence of concentration in aqueous solution can be seen. In agar the curve is slightly depressed but not displaced. The behavior of the "normal" color in nucleic acid is the same as for strongly metachromatic dyes.

Figure 13 shows the ultraviolet band of methylene blue is independent of concentration and of agar, within the limits of error.

Figure 14 shows that also in thionine the ultraviolet band depends very little on the concentration, if at all, and even on extreme variation of concentration neither the peak of absorption is displaced nor any secondary band is established.

Summary

Many basic dyestuffs are adsorbed by stainable substrates in two different shades of color, designated as the normal and the metachromatic color. As a model for a substrate stainable in the normal color, a 3% solution of nucleic acid at *p*H 4.6, is chosen; as a model for a substrate stainable in the metachromatic color a solution or gel of agar at *p*H 4.6 is chosen. These model substrates allow of spectrophotometric measurement of the absorption curve. It is shown that all dyestuffs capable of metachromasy disobey Beer's law in aqueous solution, due to the fact that with increasing concentration dimeric molecular aggregates of the dye molecules are formed. The absorption maximum of the dimer lies at shorter wave lengths than that of the monomer as it exists in extremely dilute solution. In the presence of agar still higher molecular aggregates are formed and it is these aggregates which are adsorbed by agar. The absorption bands of these high polymers are still further displaced toward shorter wave lengths and are more diffuse. In contrast, in the presence of nucleic acid the absorption spectrum of all basic dyes is independent of the concentration of the dye and is similar to, although not identical with, that of the dye in extremely dilute solution. No polymerization takes place. Each cation of the dye is combined with one acidic side chain of the nucleic acid to form a stoichiometrically well-defined salt-like compound. Very little is known about the correlation of the chemical structure of a dye with its faculty of polymerization. On the other hand, this faculty of polymerization is always correlated with the metachromatic effect.

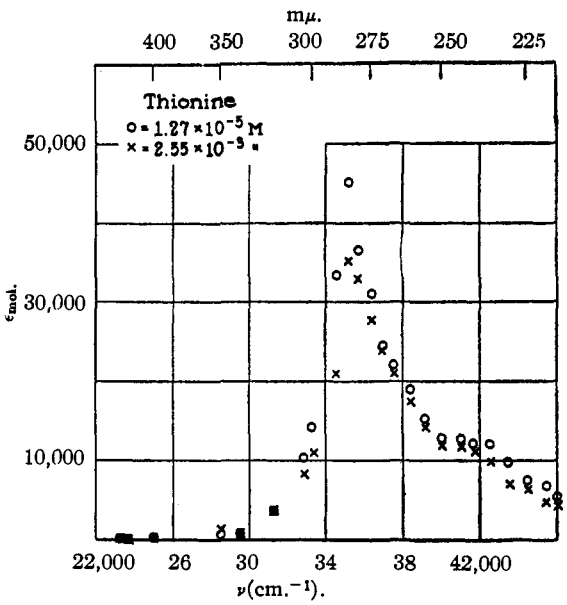

Fig. 14.

NEW YORK, N. Y. RECEIVED MARCH 23, 1945

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY No. 1003]

The Serological Properties of Simple Substances. X. A Hapten Inhibition Experiment Substantiating the Intrinsic Molecular Asymmetry of Antibodies

BY DAVID PRESSMAN, JOHN H. BRYDEN, AND LINUS PAULING

It was shown by Landsteiner and van der Scheer[1] that by inoculating animals with suitable antigens antisera can be produced which can distinguish between optical isomers. Their first experiments were carried out with antisera prepared with use of immunizing antigens made by coupling proteins with diazotized *d*- and *l*-*p*-aminobenzoylphenylaminoacetic acid; each of the two antisera precipitated preferentially the test azoprotein containing the corresponding haptenic group, and the precipitation was inhibited preferentially by the corresponding hapten. Similar results were also obtained with azoproteins containing azo derivatives of the stereoisomeric tartranilic acids as haptenic groups.

These experimental results show the significance of spatial configuration in serological reactions. They do not, however, depend in any way on the fact that antibodies themselves have inherent optical activity. Because of the optical activity (*l*-configuration) of the amino acid residues of proteins, the possibility exists that an antiserum made by use of an antigen prepared from an optically inactive haptenic substance may combine preferentially with one of a pair of optically isomeric substances. We have prepared such an antiserum (anti-S_p serum), by injecting rabbits with an azoprotein made from the inactive

(1) K. Landsteiner and J. van der Scheer, *J. Exptl. Med.*, **48**, 315 (1928); **50**, 407 (1929).